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Emergent Complexity of Microbial Communities in the Planetary Crust

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Doctor of Philosophy
The University of Edinburgh
2018

Abstract

Microbial communities are highly complex systems, yet are assembled from basic building blocks of some of the simplest organisms on Earth. We currently have ample information on many individual microbial taxa, but we lack fundamental understanding of how complexity emerges as microbial communities are assembled. As microorganisms almost always exist in complex communities, a series of experiments were implemented in order to study the factors involved in community assembly. Here, focus was placed on investigating two assembly processes described by the metacommunity concept: neutral assembly, dominated by stochastic processes, and species sorting, where the environment selects for the emerging complex community. The process of assembling a complex microbial community on different rock substrates was studied in a series of interlinked experiments. In an experiment examining colonisation of two end-member igneous rock types over the course of 1.5 years, it was hypothesised that neutral processes would dominate at the outset, with environmental selection and thus species sorting becoming more important with time. The results indicate that the opposite is true: the communities are selected for at the outset and converge through neutral processes to a more complex community as the environments become more similar over time. Other experiments were set up in order to probe different factors controlling the assembly of complex microbial communities. Microbial environmental engineering was studied by investigating microbially-mediated rock weathering and its effect on the emerging community. The role of priority effects in building a complex community from simple building blocks was investigated using strains isolated from the colonisation experiment, by mixing together single isolates with some time lag into a co-culture. Lastly, the impact of environmental perturbation on viability of communities at different stages in the assembly process was studied using stresses such as freeze-thaw and desiccation. Together, these experiments have given greater insight into the various factors that influence the assembly of a complex microbial community.

Lay Summary

Microorganisms are the most versatile and widely distributed organisms on the Earth. Microscopic bacteria, fungi and other tiny organisms can be found in virtually every environment on this planet, including ones that would be inhospitable to any other life forms. Because of their small size and remarkable capabilities to adapt to new environmental conditions, microorganisms can readily colonise almost any surface or habitat they come across. Almost all of these habitats contain complex communities of different microbes that inhabit the same space and share and exchange resources in their bid to grow and survive. Sometimes, new pristine environments are created after various sterilisation processes, such as natural events like volcanic eruptions or wildfires, or by human action such as cleaning with a disinfectant or heating an object to a very high temperature, whereby the current resident microorganisms are killed off. When these sterilised habitats or surfaces are created, new microorganisms readily and rapidly take up residence, as they are brought into these new habitats by dispersal through the air, or by being brought into direct physical contact through the action of other organisms or inanimate processes. Microorganisms subsequently survive under the new conditions by employing their diverse abilities to utilise resources and adapt to new environments.

Many microorganisms are remarkably well suited for colonising and living on rock surfaces. Despite their rather barren appearance, rocks can be a good home for microorganisms, by providing a smorgasbord of nutrients that can be leached from its various constituent minerals, and giving its tiny residents a solid surface on which to live and thrive. At the outset however, an exposed rock surface can be a hostile environment, without many sources of energy to use for growth and survival, and subject to extreme fluctuations in for instance temperature, humidity and radiation. The pioneer organisms to colonise a new surface have to be able to survive on these limited resources under what can sometimes be

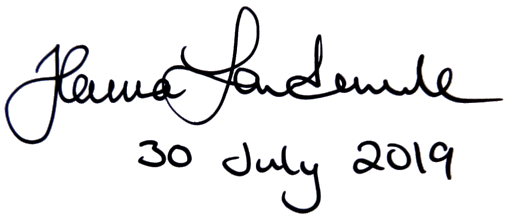
harsh, exposed conditions, before their presence, through their metabolism and life cycle, helps create a more lush environment where new organisms can survive. In this way, an initially simple community, consisting of just one or a few species, becomes a complex community as more and more species take up residence. This community continually alters and breaks down the rock, leading to further changes in the community depending on which organisms can survive as the environment changes. Over time, the interaction of microorganisms with rocks and minerals leads to the formation of various soils and clays, on which the rest of Earth's life forms, humans included, depend.

In this project, the process whereby a microbial community is formed in a new environment was studied through a series of experiments. The first of these experiments was a long-term study in how microorganisms colonise new rock environments from the atmosphere through the actions of wind and rain, by placing tubes of sterile, crushed rocks outside. The experiment was conducted over 1.5 years, and it was found that the microbial communities changed continually throughout the course of the study, and that the type of rock substrate influences the structure of the microbial community, in that different rock types allow for different organisms to thrive. In the second experiment, the ability of microorganisms to survive environmental stress, in terms of freezing and thawing cycles, at various points during the process of community assembly and colonisation was examined. It was found that microbial communities are better at withstanding changes early on in the community assembly process, when many hardy pioneer species are present. In the third experiment, the effect of priority in community assembly was examined, whereby different organisms were added to the new environment at different times. It was found that an early arrival into a new environment gave organisms a clear advantage in growth and survival over species entering the system at a later stage. Lastly, the processes by which microorganisms can leach elements from rocks were studied, and it was concluded that the leaching patterns are dependent on the type of rock substrate, and whether or not oxygen is present such that the organisms can undergo aerobic respiration, and that these leaching patterns help shape the resident microbial community.

Taken together, these experiments reveal several features of how microbial communities are formed in new environments, and significantly increases our knowledge of microbial colonisation and community assembly processes with possible implications for a wide range of environments.

Declaration

I declare that this thesis was composed by myself, that the work contained herein is my own except where explicitly stated otherwise in the text, and that this work has not been submitted for any other degree or professional qualification except as specified.



(*Hanna Landenmark, 30 July 2019*)

Acknowledgements

This project has been a true joy to undertake, and I feel incredibly blessed and thankful to have had this opportunity. I owe the successful implementation and conclusion of this story to some special people, whom I would like to express my sincere thanks to here.

I would firstly like to thank my primary supervisor Charles Cockell for the opportunity to undertake this project, and for expert guidance and advice throughout the process. Working with Charles has been incredibly rewarding, and his vast knowledge of the wider subject area has been truly invaluable. Moreover, his vision and approach to science and life's biggest questions, as well as his can-do attitude, has been an endless source of inspiration and really pushed me to think further and grander. Thank you Charles, for everything you have taught me and the support you have offered me.

I would like to thank my second supervisor Rosalind Allen, for constant support and encouragement. Rosalind's pragmatic and knowledgeable approach to the research questions at hand was central to the success of this project, and I count myself very lucky to have been able to rely on her for direction and advice.

I would also like to thank my annual reviewers, Cait MacPhee and Job Thijssen, for their feedback and guidance at regular intervals throughout this project, as well as useful careers advice and support.

None of this would have been possible without the support of my lab group, a large group of people who have all at one point or other offered help and advice, as well as endless laughs and companionship. It was a real pleasure to work in such a dynamic, knowledgeable and fun group, and I am very thankful for all the help around the lab as well as advice on PhD life in general. My sincerest thanks to Mark Fox-Powell, Sam Payler, Toby Samuels, Petra Schwendner, Susana Direito, Jesse Harrison, Sophie Nixon, Casey Bryce, Natasha Nicholson, Jennifer Wadsworth, Delma Childers, Adam Stevens, Claire-Marie Loudon (McLean), Rosie Cane, Liam Perera, Andy Dickinson, Ryan Hunt, Pete Higgins, Prarthana Desai, Yuchen Shang and Sean McMahon. My sincere thanks to you all, without whom I definitely wouldn't have stood a chance.

Special thanks also go out to those who at some point or other helped me with

fieldwork, often under blizzard-like conditions of wind, rain and snow, even if it was just on the roof of our building: Charles Cockell, Toby Samuels, Ana Fialho, Katy Dickinson and Adam Stevens. Thanks to Charles for hilariously pointing out that sampling on the roof of the JCMB reminded him of fieldwork in the Arctic. It was miserable indeed.

Big thanks go out to the many technicians and experts from various fields who helped with various parts of the project. Thanks to Scott McLaughlin for help with the box design and set-up for the colonsation experiment. Thanks to Lorna Eades at the School of Chemistry, The University of Edinburgh, for help with ICP-OES and ICP-MS. Thanks to Nick Odling of the School of Geosciences, The University of Edinburgh, for help with rock crushing and XRF and XRD analysis. Thanks to Ruth Monfries at the Royal Botanic Garden Edinburgh for providing the weather data for the duration of the colonisation experiment.

Special thanks go out to Lisa Wink and Christine Moissl-Eichinger at the Medical University of Graz for expert help with the qPCR analysis.

A group of people who shall receive thanks not because of their scientific integrity (although you are all, of course, brilliant scientists in your own right) are my dear, dear friends that I have shared an office with for the duration of our PhD studies. The warmest of thanks go out to Katy Dickinson, Carolina Pereira da Silva, Ana Fialho and Yair Fosado, as well as honorary office member and neighbour Martina Foglino. I can say with certainty that without your friendship and all the fun we had together, none of this would have been possible. Thanks for the constant support you have offered me. I will truly miss our conversations about everything from under the sun, from music and movies to politics and faith. Some days, when all the science was going terribly wrong and on many bleak and cold winter mornings, I was still looking forward to coming into work and seeing you all, and you always brightened my day. Thank you for being the best companions a PhD student could ever have.

I would like to thank my family - mother Barbro, father Lennart and brother Johan. Somehow, we have ended up being a family where everyone found their way to studying biology in some way or another. Thank you for raising me to be a hard-working individual and always encouraging me to try my hardest in school and beyond. Thank you for inspiring my love for the natural world and the outdoors by taking me on countless adventures looking for rare plants and birds. I like to think that my love for of science and all creatures great and small is just an extension of the seed that you sowed with an overall reverence for nature. I think that, in many ways, coming from a family of scientists made this journey very natural to me, and I am always grateful for your support and encouragement. Extra thanks also to my mother and maternal grandmother, Ella, for being inspiring women and pioneers who made me always take for granted that women could do anything and be whoever they wanted to be.

Finally, I would like to thank my long-suffering partner-in-crime and husband Dave Hammett for his endless love and support. I know that you know better

than anyone else what a roller-coaster journey this has been, and you were always the first to pick me up when I fell as well as celebrate with me when progress was made. Your enthusiasm for and left-field take on my project was central to many new insights, and for that I am very thankful. Thanks also to Pebble the hamster and Cusco and Nazca the guinea pigs. My little canal-side family and home continually offers a refuge of love and warmth and inspires me to always be the best that I can be.

Last but not least, I thank God for my life and for this wonderful Earth and all that is in it.

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Chapter 1

Introduction

The interactions between life and the geosphere are complex and fundamental to many Earth processes and to life itself, but are often poorly understood and modelled. Yet they play a crucial role in biogeochemical cycling, defining the limits to life on Earth and the flow of energy through Earth's ecosystems. In this project laboratory experiments and outdoor microcosms are used to understand the processes by which microorganisms colonise rock environments, and the catalytic rates of alteration and extraction of energy and nutrients from rocks by life compared to abiotic conditions. Colonisation is studied through a natural system where microorganisms enter a new environment, and thus build up a complex ecosystem from simple primary components. These experiments involve the use of natural communities and isolates to investigate how organisms under aerobic and anaerobic conditions react with and accelerate the break-down of natural substrates. The results are coupled to theoretical models of rock-biosphere interactions using the laboratory data to constrain these models. This work will not only advance our understanding of biology-geosphere interactions, but will improve our ability to predict and interpret complex biological systems.

Interactions between biology and geology are fundamental to the history and future of the Earth [152], [51], [96], [114]. Among the most notable areas is global nutrient cycling, which enables and sustains ecosystem function. Living organisms can mobilise nutrients which can be moved through different mechanisms to new locations where they can be taken up and utilised by other organisms [247]. Microorganisms play an active role in soil formation by breaking down rocks into fine grains that over time form the soil matrix,

and aid precipitation of minerals and formation of calcite and carbonate [247]. Significant interaction between biology and geology is observed in the deep subseafloor, where marine sediments form the largest global reservoir of organic carbon and cell turnover takes place on nearly geologic timescales [195]. A familiar example of putative microbial interaction with rock relevant to the early Earth is stromatolites, which are layered structures formed by succession of volcanic rock with microbial sedimentation [3].

Biosphere-geosphere interactions have several important areas of application to modern society. Firstly, a better understanding of microorganisms in different environments has important implications for most areas of life where microbes are encountered, and is especially important for healthcare, as disease can both be mediated and mitigated by microorgansisms. Secondly, increased understanding of soil formation and geochemistry has applications for agriculture and industry across the globe. Thirdly, the biological implications for water geochemistry and erosion can be used to inform planning for vulnerable habitats and ecosystems. Lastly, findings on biological weathering can feed into climate models and bioremediation endeavours, as understanding natural climate stability is of paramount importance in assessing the anthropogenic contribution to climate change. Microbial activity might play a significant role in regulating the Earth's climate through its part in the carbonate-silicate cycle. In sum, this interdisciplinary field has far-reaching implications on both local and global scales.

An important implication of studying biosphere-geosphere interactions is that they are, with our current knowledge, exclusive to the Earth. No other planet is at present known to harbour or have harboured life, and hence the Earth is unique from a planetary science perspective. By probing the complexities of the relationship between biology and geology we are creating a framework for understanding how the Earth might differ from other planets in our solar system and beyond.

Microorganisms interact with the geosphere through the weathering of rocks. Weathering refers to any process whereby rocks are broken down *in situ* and is carried out by mechanical, chemical and biological agents working in concert. Weathering is often coupled with erosion, which physically moves weathered rock fragments to new locations. Through the process of rock and mineral weathering, microorganisms such as bacteria and archaea facilitate the release of nutrients from rocks which can be taken up by other organisms, such as plants

and fungi, making microorganisms fundamental agents in many ecosystems, and are particularly important for soil formation. Remarkably, microbe-mineral interactions can be both detrimental and beneficial to society, depending on the circumstances and environments in question. Examples of generally detrimental microbial weathering is tooth decay and break-down of mineral-based historic monuments and artefacts, whereas biomining and biological fertilisers are counted as useful applications of microbial weathering abilities.

Interactions between microorganisms and minerals form an intricate feedback system between weathering and the emergence of complexity in microbial communities. Microorganisms that colonise fresh rock surfaces have to acquire most of the nutrients they need for growth from either the atmosphere or the substrate, where their effect on the rock substrate causes the rock to weather. By breaking up the rock either as an active process in order to acquire nutrients, and through the build-up of metabolic byproducts, the local abiotic environment and geochemistry is altered. These environmental changes, as observed by elemental leaching and alterations of the geochemistry, as well as the build-up of organic carbon, can benefit species that could not previously inhabit the unaltered surface or habitat, but can take up residence as a result of the altered circumstances. As more species start to colonise the surface or rock habitat, a complex community emerges, in which different species perform different functions. The complex community continues to further weather the rock, generating new leaching patterns, creating a dynamic habitat where resources are utilised and recycled within the ecosystem. At the same time, weathering increases as novel metabolisms dissolve more nutrients from the rock that then become available to the community. Hence, an initially simple system with limited constituents evolves into a complex construct. In this way, community development is both a product of and a catalyst for weathering. Disentangling this feedback process involves studying both weathering and community assembly in novel mineral environments.

The colonisation of volcanic rocks has implications for understanding past and present Earth processes, with parallels between the first land organisms and current pioneer species which first inhabit new landmasses. Rocks can be regarded as primary ecosystems, which offer foundations for life that only particular organisms can utilise and adapt to. These first organisms need to survive harsh, nutrient- and organics-poor conditions, which can be subject to repeated dessication, high radiation doses and temperature fluctuations. Most studies

on colonisation are conducted on volcanic rocks on timescales from months to often years after deposition, hence more work is needed to understand the first colonisers, and to understand better how the colonisation process changes with time through more rigorous time-series sample collection. Other related studies have looked at microbial community assembly in various sterile environments, such as infant guts, pitcher plants, re-wetted soils and overlaying water-column of dried sediments, or model environments such as rock pools and Winogradsky columns.

Currently, there is a lack of understanding of certain key aspects of microbe-mineral interactions. Firstly, there are gaps in the knowledge regarding the processes involved in and the effects of microbial weathering on different parts of the Earth's crust. In addition, there is limited appreciation of how microbial communities become established and colonise new environments, especially rock environments that are abundant on the Earth. These issues are closely linked through a feedback process between microbes and minerals, in which rock weathering is both influenced by and affects community assembly. Microorganisms colonising a fresh rock surface will weather the rock by breaking down the substrate, causing nutrient release. These nutrients can be utilised both by the pioneer colonisers and new species that take up residence on the surface, leading to the emergence of a microbial community, which further weathers the surface. In this way, it is important to advance the knowledge of both weathering and community assembly and the feedback processes involved. The nature of these processes requires the investigation of how an initially simple system with few constituents develops into a complex community.

In this project, certain specific aspects of biosphere-geosphere interactions are investigated, which tie in with major events in the Earth's evolutionary history. Laboratory experiments are coupled with studies on outdoor microcosms as well as computer modelling in order to approach the process from different angles. Even with existing comprehensive experimental investigations, there are fundamental gaps in our understanding of how complex microbial systems become established. Field and laboratory experiments are generating invaluable data on colonisation in different environments, whereas computer simulations help probe highly complex systems and environments which are difficult to access, or to study the process on timescales that are not conducive to laboratory experiments. In sum, a dual approach is needed, whereby experimental, field and modelling data are used in concert in order to advance the subject. The two major themes under

study are microbial rock weathering and colonisation of early and newly formed landmasses, both of which relate to the way in which microbes interact with and utilise different rock substrates and how microbial communities are assembled. Attaining empirical data on these interactions at a small scale can hence shed light on some large-scale, global phenomena linked to the past and continuing evolution of the Earth.

All work undertaken in this project is based on top-level questions regarding unravelling the complexity in biosphere-geosphere interactions and microbial community assembly that have not yet been fully answered:

- How does the planetary crust influence the emergent complexity of microbial communities, and to what extent are some parts of the planetary crust more susceptible to microbial weathering?
- What factors influence microbial community assembly in new environments?
- How important are priority effects in community assembly of microorganisms colonising new habitats?
- How does environmental stress during colonisation affect the emerging complex community?
- How do pioneer microbial communities change or enhance habitability for later colonisers?
- How would major planetary change, such as the oxidation of the atmosphere, affect microbial weathering?

The aims of this project was to study biosphere-geosphere interactions pertaining to microbe-rock processes. The initial part, chronologically, of the study was concerned with the global carbonate-silicate cycle and trying to address the question of how microbial rock weathering contributes to this. In addition, the initial experiment also compares and contrasts microbial rock weathering under aerobic and anaerobic conditions, which has relevance for the Earth's history as well as different environments today. Secondly, experiments and modelling were conducted within the topic of early colonisation of newly formed landmasses such as volcanic rocks, addressing questions such as how these early communities are formed and change, through a series of interlinked experiments. The two

parts of the project help elucidate the feedback process by which complexity emerges in microbial systems, in which weathering both affects and is influenced by the establishment of a complex community and helps inform about biosphere-geosphere interactions on both local and global scales.

The first experiment was set up as a study of the weathering of granite and gabbro by a natural community of organisms taken from soil samples collected from corresponding areas of volcanic rocks on the Isle of Skye, Scotland. Utilising two different types of igneous rocks enables investigation of how substrate affects the ability of a complex microbial community to weather rocks. Rocks with high silica content, such as granite, are more stable at the Earth's surface and tend to weather slower than rocks with lower silica concentrations, such as gabbro. The experiment was also intended to compare and contrast weathering under aerobic and anaerobic conditions. The set-up consisted of flasks with crushed rock, a minimal glucose-based weathering medium and a soil inoculum.

The second phase was set up to examine how complex microbial systems are established and study the early colonisation of newly formed landmasses, through a joint effort utilising outdoor microcosms, modelling and laboratory experiments. The main experiment consisted of outdoor microcosms in the form of flasks with sterilised rocks that were inoculated from the atmosphere through wind and rain. This experiment was run for 1.5 years, with sacrificial time points every 1-2 months allowing the emerging complex community and changing environmental variables to be monitored.

Two more experiments were set up to study different aspects of community assembly - priority effects and environmental stress. Both of these experiments were conducted in the laboratory using microcosms and individual strains of bacteria that had been isolated from the colonisation experiment, in order to elucidate some of the factors that may be influencing the community assembly in the natural system.

In sum, this project aims to study the complex relationship between microbes and minerals. An increased understanding of rock weathering under different conditions will help elucidate how microbial communities are established. Similarly, by studying community assembly, insight can be gained into community function and how this impacts the continued dissolution of the Earth's crust. This study investigates the relationship between weathering and community assembly, shedding light on a fundamental biosphere-geosphere interaction.

Chapter 2

Background Literature Review

2.1 Biosphere-Geosphere interactions

Interactions between biology and geology are fundamental to the history and future of the Earth. Since life began on the Earth, it has inevitably and irreversibly altered the course of the evolution of the Earth's geology. Both nutrient distribution and mineralogical composition would be vastly different had life never originated and made its mark on this planet. Biology has played many important roles in shaping the Earth system, such as oxygenation of the atmosphere, the formation of soil and the composition of many rocks and minerals. Many aspects of the biosphere have an impact on the Earth's geology, but it is often most pertinently observed when considering microorganisms, which often interact directly with rocks or minerals through their metabolism on exposed rock surfaces, in the deep subsurface or in aquatic environments such as lakes and oceans. Although microorganisms are the Earth's smallest creatures, they are incredibly versatile and prevalent in nearly all types of environments on the Earth. Direct evidence of their engineering capabilities can be seen in for instance stromatolites, stratified structures of sedimentary rock shaped by biofilm layers of cyanobacteria formed by succession of rock with microbial sedimentation [3], the weathering and breakdown of historic monuments [234], and the many coloured rivers and lakes around the world, such as Rio Tinto, Spain and Grand Prismatic Spring, USA [202]. Understanding the intricacies of the interaction between the biosphere and the geosphere are hence fundamental to understanding the Earth system and addressing future environmental challenges.

Among the most notable areas of microbial interaction with the geosphere is global nutrient cycling, which enables and sustains ecosystem function. Living organisms can mobilise nutrients which can be moved to new locations through different mechanisms where they can be taken up and utilised by other organisms [247]. Microorganisms play an active role in soil formation by breaking down rocks into fine grains that over time forms the matrix of the Earth's soils, and aid precipitation and formation of various minerals [247]. Significant interaction between biology and geology is observed in the deep sub-seafloor, where marine sediments form the largest global reservoir of organic carbon [195]. Thus, soils and sediments as we know them on the Earth would not be present without the action of biological agents such as microorganisms.

Biosphere-geosphere interactions have several important areas of application to modern society. Increased understanding of soil formation and geochemistry has applications for agriculture and industry across the globe. Findings on biological weathering can feed into climate models, from an increased understanding of carbon cycling, and bioremediation endeavours, from understanding how microorganisms can take up and alter heavy metals in the environment. The biological implications for water geochemistry, erosion and soil formation are important for understanding many vulnerable habitats, and alterations in these processes often have a direct impact on human life and society, in for instance water quality, coastal erosion and the degradation of soils in agricultural settings. In sum, this interdisciplinary field of study has far-reaching implications for human life and the rest of the biosphere on both local and global scales.

Biosphere-geosphere interactions are, as far as we know, exclusive to the Earth. The Earth is unique from a planetary science perspective, in that it is the only known planet to have ever hosted life. The geological processes on other celestial bodies sometimes closely mimic what we observe on Earth, but can also be vastly different, where the differences seen on the Earth may be mediated both by different abiotic factors, as well as influence from the biosphere. As the Earth system has been shaped significantly by the presence of biological life for the past 3.5 billion years, the evolution and future of this planet differs greatly from that of any other known planetary body. By probing the complexities of the relationship between biology and geology we are creating a framework for understanding how the Earth might differ from other planets in our solar system and beyond, and helps us better understand the nature of life itself.

A pertinent example of biosphere-geosphere interaction is microbial weathering,

which feeds into the global carbon cycle. Understanding natural climate stability is of paramount importance in assessing the anthropogenic contribution to climate change. Microbial activity might play a significant role in regulating the Earth's climate through its part in the carbonate-silicate cycle. Urey (1952) [246] first proposed that there is an intimate link between atmospheric carbon dioxide levels and the weathering of silicate rocks. Weathering of rocks releases cations that can bind atmospheric CO_2, depleting CO_2 levels in the atmosphere. The rocks are subducted into the Earth's mantle on geological timescales, after which CO_2 is released back in the atmosphere through volcanism. The balance between weathering and volcanism determine the instantaneous concentration of atmospheric CO_2 [25]. The weathering of rocks by microorganisms feeds directly into this process, influencing the level of greenhouse gases present in the Earth's atmosphere, and therefore impacts Earth's climate.

Another major event in the Earth's history with relevance to microbiology is the Great Oxidation Event (GOE). The early Earth had an atmosphere with minimal levels of oxygen, however, at approximately 2.4 Ga, a rapid change took place in which oxygen levels rose to concentrations of a few percent of Present Atmospheric Level (PAL) [216]. Central to this change was the emergence of organisms capable of oxygenic photosynthesis, whereby water could be used as an electron donor for energy acquisition in the making of adenosine triphosphate (ATP), resulting in a drastic increase in oxygen. Aerobic modes of energy production generate up to ten times more energy than the pre-existing anaerobic methods, giving organisms capable of aerobic energy acquisition a distinct evolutionary advantage, as they can grow much faster. With this change, the ability of microorganisms to weather rocks and the amount of nutrients in the biosphere also increased significantly. The simultaneous increase in elemental release from weathering at the GOE would have fed directly into the Earth's climate cycle, making it an important time point in understanding the Earth's evolutionary history.

Microbial weathering has several useful applications to society, where increased understanding of the phenomenon can lead to advances in both industry, agriculture and healthcare. Through the process of weathering, microbes facilitate the release of nutrients which can be taken up by other organisms, such as plants, fungi and other microorganisms, making them fundamental agents in many ecosystems. Microbial weathering is responsible for a range of detrimental phenomena such as tooth decay, break-down of mineral-based historic monuments and buildings, and have a role to play in accelerating coastal erosion. Conversely,

there are several ways in which microbial mineral weathering can be utilised for the benefit of society. For instance, the nutrient-releasing capabilities of microorganisms make them an ideal candidate for biological fertilisers, which have the potential to be more cost-effective and environmentally friendly than many chemical fertilisers [247], [109], [241], [37]. Microbes can also be utilised in biomining to leach out specific minerals from ores [205, 214, 247]. In fact, biomining has become so important in certain circumstances that for instance about 15% of copper and 5% of gold being mined is now extracted with the help of microorganisms [138]. Thus, it is important to understand in detail how microbe-mineral interactions occur, both to utilise them for the benefit of society, as well as mitigating detrimental effects.

2.2 Microbial colonisation and life in rock environments

Microorganisms are the first forms of life to colonise rocks when new rocks are formed, such as after a volcanic eruption, paving the way for other forms of life to subsequently take hold. This type of habitat and interaction between microorganisms and rocks is also likely one of the earliest ways of life on the Earth, when most of the Earth's terrestrial surface was rocky and the only creatures in existence were ancient forms of microorganisms [114]. Rocks in almost every environment that is barren of other vegetation or life hosts some kind of microbial community, even in harsh and fluctuating conditions, such as the growth of hypolithic communities growing on the underside of translucent rocks in polar environments [56]. Today, the process of colonisation is rapid, thanks to the Earth's vigorous hydrological and atmpspheric cycles, giving rise to ubiquitous rock-dwelling microbial communities on this planet [53].

2.2.1 Microbial ecosystems inhabiting rock environments

Microbial ecosystems living in or on rocks are present in most environments on the Earth, provided that there are no other extreme conditions such as temperature, radiation levels etc., that otherwise push the environment beyond the limits of life. Still, life on rock often sits at the limits of life, with high levels of exposure to radiation and the elements [9]. If the overall conditions of the site are mild,

the rocks are usually also colonised by macroscopic vegetation, whereas if the conditions are harsh, microorganisms will remain as the only colonisers [114]. Often, microorganisms can be found both on the rock surfaces (epiliths) and inside the rock (endoliths), given that cavities exist or can form to allow for colonisation [111]. Examples of environments include both natural cases such as cliff faces, lava flows and exposed rocks and sand, and man-made environments such as buildings, monuments and paving. In some cases where microorganisms colonise man-made structures it can have a detrimental effect on the structures themselves, such as discolouration or etching, for instance by green algae, or even compromising the structural integrity of the monument or building [66].

Depending on the substrate and other environmental and dispersal factors, these communities can vary widely, but there are certain common challenges and benefits to living in this niche. Firstly, species must be able to acquire all the nutrients they need from the air or rock, or in the case of an older community, also from dead biomass on the surface. Often, single species cannot perform all the metabolic functions necessary to alone colonise a sterile, organics-free surface, but a community working in concert can ensure the survival of its members [114]. Secondly, species must be able to attach to the rock surface, for instance by forming biofilms or mats. Thirdly, the community must be able to withstand significant environmental stress, such as fluctuating temperatures, desiccation and rewetting, radiation, salt stress etc. [114]. Depending on the location and type of rock environment, these stressors will be more or less prevalent. These factors mean that organisms and communities on rocks may not be identical, but must be equipped to survive shared complications.

The types of organisms present in a rock-dwelling community are typically algae, bacteria, fungi and protozoa. The first species to settle on exposed rock tend to be chemoorganotrophs that acquire minerals and organics from the air. Heterotrophs can also be found as pioneer species, or as an important or dominant part of a more established community. Sulfur and nitrifying bacteria are also commonly found. Cyanobacteria are well-known and studied denizens of rock environments, where they provide a carbon source for other organisms through the process of photosynthesis. Among the most common heterotropic bacteria in rocks identified through cultivation are *Arthrobacter* sp., *Bacillus* sp., *Micrococcus* sp., *Paenibacillus* sp., *Pseudomonas* sp. and *Rhodococcus* sp. Through molecular methods, the main phyla often found are Proteobacteria, Actinobacteria, Acidobacteria and the Cytophaga – Flavobacterium — Bacteroides group [114]. There are many

different studies examining the content of rock-dwelling microbial communities, and there are both differences and similarities in community compositions of the studied environments.

2.2.2 Microbial colonisation of novel environments

Microorganisms such as bacteria and archaea are incredibly versatile and as such are present in almost every habitable environment on the Earth. In large part thanks to the Earth's vigorous hydrological and atmospheric cycles, newly formed habitable environments become rapidly colonised by new organisms that can thrive under these conditions, as the uninhabited habitats are swiftly linked with inhabited environments that already host life. On the Earth, uninhabited habitats are thus very rare [53]. Several studies have looked at microbial community assembly or re-assembly in various sterile environments, such as infant guts, pitcher plants, re-wetted soils and the overlaying water-column of dried sediments [160].

2.2.3 Microbial colonisation of rock environments and lava flows

The colonisation of newly formed volcanic rocks is a process which has implications for understanding past and present Earth processes, with parallels between the first land organisms and current pioneer species which first inhabit new rock environments. New lava flows get colonised very quickly, on the order of days and months [145]. Pioneer organisms pave the way for soil formation and ecosystem development, for instance through their role in weathering the rock. These first organisms to colonise new habitats need to be able to survive harsh, nutrient- and organics-poor conditions, which could be subject to, for instance, repeated dessication, high radiation doses and temperature fluctuations [145]. Rocks can be regarded as primary ecosystems, which offer foundations for life that only particular organisms can utilise and adapt to [247]. To the pioneer organisms belong phototrophs, chlorophytes, lichens and mosses [247], [145]. Among the first microbial species found on recent volcanic deposits are Acidobacteria, Alphaproteobacteria, Betaproteobacteria, Actinobacteria and Cyanobacteria [145]. However, most studies on colonisation are conducted on volcanic rocks years after the lava has been deposited, hence more work is needed

in order to understand the first colonisers [145]. In addition, no study has so far been conducted where the process of colonisation of fresh volcanic rock is monitored with a rigorous time series sampling, thus recording the process of colonisation over defined time scales. The work conducted in this project bridges that gap.

A study of the lava flow at Fimmvorduhals, Iceland after the Eyjafjallajokull eruption in 2010 showed that the microbial community was dominated by Betaproteobacteria, specifically *Polaromonas*, *Variovorax* (Comamonadaceae) and *Duganella*, *Herbaspirillum* and *Massilia* (Oxalobacteraceae) species [145]. The lava flow was sampled twice at 3 and 5 months after the eruption, and the microbial community was studied both through culturing and DNA sequencing.

Actinobacteria have also been found in significant abundance in volcanic glass [54]. Specifically, samples of obsidian, weathered basaltic glass and crystalline basalt were collected in Iceland and crushed before being analysed through 16S rRNA sequencing and cultured by scattering rocks on agar plates. The basaltic glass was found to be dominated by Actinobacteria, which also made up a considerable proportion of the crystalline basalt and obsidian samples. The other most common taxa were Proteobacteria, Acidobacteria, Bacteroidetes and Cyanobacteria in basaltic glass; Proteobacteria, Acidobacteria, Bacteroidetes and Verrucomicrobia in crystalline basalt; and Proteobacteria, Acidobacteria and Cyanobacteria in rhyolitic obsidian.

In a similar study of Icelandic rhyolitic glass, the community was again analysed through crushing the rock and performing a DNA extraction. The community was found to consist of Actinobacteria, Acidobacteria, Verrucomicrobia, Alphaproteobacteria, Cyanobacteria and Planctomyceta. The study found that the weathering patterns match those of the slower chemical weathering process expected for rhyolite compared to basalt [123].

A study on hypolithic communities on desert rocks from 92 deserts worldwide has revealed that these communities are primarily dominated by Cyanobacteria to about 75%, followed by Alphaproteobacteria at about 10-20%, and smaller fractions Acidobacteria, Actinobacteria and Bacteroidetes. The remainder of the community is made up of Betaproteobacteria, Gammaproteobacteria and Chloroflexi. The Cyanobacteria is found to be primarily Oscillatoriales and Pleurocapsales (*Chroococcidiopsis* spp.) [44].

There is additional support for the fact that Actinobacteria, Proteobacteria,

Bacteroidetes and Acidobacteria dominate the microbial community in natural volcanic rocks [55], [57]. A study by Cockell et al. [55] find these to be the dominant phyla in a natural weathering environment in Iceland consisting of basaltic glass and palagonite subglacial (hyaloclastite) deposit. Some selected isolates grew well at circumneutral pH, but growth was retarded by lowering the pH. A similar phylogeny was found at the same site by Kelly et al. [144], with a nearby rhyolitic glass being inhabited primarily by Cyanobacteria, Proteobacteria, Acidobacteria and Actinobacteria.

Another related study by the same research group has compared the microbial diversity of two Icelandic lava flows containing crystalline rock in the same volcanic region and of similar age, one with rhyolitic composition and one with basaltic composition [143]. They found a strong correlation between the communities and the mineralogy at each site, and a higher microbial diversity at the rhyolite lava flow. Proteobacteria dominated the rhyolite lava flow while Acidobacteria was dominant at the basaltic lava flow. Clones in the dominant phyla were found to be similar to soils from Hawaii, the Andes and Antarctica, but the communities also contained a significant portion of novel strains.

The microbial communities on tombstones has been analysed in an attempt to garner understanding of the spatial variation in microorganisms around the world [33]. In a study by Brewer and Fierer [33], the microbial communities of 149 different tombstones on three continents erecte from the 1800s onward were analysed. The stones were mostly classified as either granite or limestone in composition. The communities were found to be dominated by Proteobacteria, followed by Cyanobacteria, Bacteroidetes, Actinobacteria and Acidobacteria. A few genera were shared across all samples. Correlations were found between climate and community structure, with clear differences seen between tropical and temperate regions, and temperature and mean annual rainfall taken together appear to have an influence. Rock type was also found to significantly impact the microbial community, with the granite and limestone stones hosting different communities. Direct comparison between two cemeteries containing both types of tombstone indicate that rock type has a larger influence than geographical location on the community structure. In general, the granite communities had more functional genes relating to chemotaxis and acid tolerance, while the limestone communities had more functional genes related to radiation tolerance and photosynthesis. These results illustrate the influence of rock substrate and climate on microbial communities, as well as advancing the understanding of the

global distribution of microorganisms.

Microbial colonisation of lava has been studied in relation to SO_2 exposure after a volcanic eruption, in the case of the Miyake-jima site of the Mount Oyama eruption in 2000 [94]. Ash samples were collected after 3.5, 6.6 and 9.5 years after the eruption, and the chemical environment was described, along with the succession observed in the microbial community. It was found that after 3.5 years the community was dominated by *Leptospirillum*, belonging to the phylum Nitrospirae, and *Acidithiobacillus*, belonging to the Proteobacteria, both of which decreased in relative abundance in the 6.6 and 9.5 year old samples, and that the dominant species in these genera are obligate autotrophic acidophilic nitrogen-fixing bacteria. The communities were further made up of Proteobacteria, Acidobacteria and other phyla in smaller relative abundances. The study found no presence of phototrophs in the community, which they assume to be due to the high levels of SO_2 present. The study concludes that the succession patterns in this environment are relatively short-term, due to environmental influences continuously impacting the communities upon colonisation, as soil aggregates had not yet formed at the site.

Thorseth et al. [240] (2001) studied the microbial diversity of ocean-floor basalts at a depth of 3500m, on volcanic substrates up to 50,000 years old at the Knipovich Ridge in arctic seawater near Svalbard. Here, many organisms were found to be unique and uncultured, with closest relatives being Epsilonproteobacteria and Gammaproteobacteria, with the archaeal community belonging to the Crenarchaeota. It was found that the microbial community may have significantly altered the basaltic glass that they colonised, as evidence was found of etch marks, encrusted microorganisms, and an enhanced presence of microorganisms at glass-alteration fronts, indicating that microorganisms colonise the basaltic glass shortly after an eruption, and that the microbial community may continue to alter the geochemical environment they live in over time.

The volcanic island of Surtsey, Iceland, has received much attention as a study object of colonisation, given its unique formation and pristine properties since its formation in 1963. Marteinsson et al. [176] (2015) studied the microbial community at Surtsey in 2009 through a series of surface soil samples and a 160m-deep drill hole. They found the lowest number of cells in bare pumice, but that vegetated soils and nutrient-rich environments with bird droppings hosted a larger microbial community in terms of cell counts. The amount of organic matter in the various environments was found to be correlated with the number of

heterotrophic bacteria observed. The drill holes were found to contain uncultured bacteria and archaea, with Methanobacteriales and Archaeoglobus being the most dominant taxa. This study helps elucidate the longer-term processes involved in microbial colonisation of fresh volcanic substrates.

It is an open question as to what extent does the rock substrate create differences in the resident microbial communities. Some work on algae colonising building stone has shown differences in the community depending on the substrate [65], as has work on colonisation of different minerals in soil microcosms [267], but more work is needed to understand this process better.

2.3 The emergence of complexity in the microbe-mineral system

Complex microbial communities are formed over time from simple building blocks. When microorganisms colonise a new habitat, this can be either done through seeding or immigration from an already complex community, or the community can start out with only a few organisms, and increase in complexity over time. In pristine rock environments, a complex community is formed through a feedback process between weathering and the community structure, starting off with a few pioneer species colonising the habitat, and changing the geochemical environment through weathering, enabling more organisms of various metabolic capabilities to take up residence. These new arrivals further increase weathering through their metabolic functions, and more nutrients are released, which are available for the community to use. In this way, community development is both a product of and a catalyst for weathering, and the community goes from a few constituent pioneer species to a complex community where resources are recycled throughout the system. The emergence of complexity in a microbial ecosystem is a topic that currently is not well understood in detail, and has implications for microbial communities in many different environments.

2.3.1 Community assembly

Microbial systems are often complex, with many agents performing a variety of functions in the community [271]. Research into microbial community

ecology is concerned with the dynamics of the integrated system, but also with community assembly processes. One of the fundamental questions relating to microbial communities, and indeed ecosystems in general, is whether community assembly is deterministic or stochastic, and hence predictable or unpredictable [192]. This question ties in with the consideration of whether organisms change environmental conditions to suit the wider community, or whether life simply adapts to a static environment [272]. In the former case, organisms affect their environment as a by-product of their metabolism, where nutrient cycling and environmental regulation is present. There are several competing theories for ecosystem development along these lines of argument.

Community assembly has been studied in natural systems, laboratory environments using microcosms and artificial communities [117], and by computer simulation, such as artificial neural networks [162]. More work is needed to better understand this highly complex topic involving countless variables and interactions.

2.3.1.1 The metacommunity concept

The processes by which new microbial communities assemble are the subject of some debate. Different studies point to distinctly different pathways for community evolution. One theory of community assembly is called the metacommunity concept [165]. According to this framework, community assembly is thought to be governed by four main processes: species sorting, patch-dynamics, mass effect and neutral processes. Each of these processes can have an impact on a developing community, either alone or in concert with any or all of the others.

Species sorting refers to a specific selection of organisms by the local environment. The environmental factors that play a role are for instance abiotic factors and competition between species, which combined select for the organisms that take up residence in the new habitat [160]. Here, the local abiotic environment is presumed to have some variation, and will affect various aspects of species interaction.

The patch dynamics perspective assumes that a community resides in a system of multiple identical patches, where the population is reduced by extinctions of both stochastic and deterministic natures. Population increases take place through dispersal. Interspecies interaction is important for both extinctions and

dispersal, and coexistence is possible in a homogeneous environment depending on competition and colonisation.

The mass effects view is centered on dispersal, and assumes that different patches have different abiotic conditions at any given time. The patches are connected such that source-sink effects can occur between populations in different patches. If this is the case, then mass effects can determine the relationship between community structure and local environmental conditions.

The alternative explanation is the neutral theory, which can be thought of as the null theory to the other three, whereby regional invariance causes the community to be assembled through dispersal factors, also known as ecological drift, which determine the rates of immigration and emigration of the system [160], [45]. The neutral theory suggests that taxa and species that are widespread and abundant will be common on both regional and local scales. Hence, the community in a specific environment will be determined by rates of immigration and emigration. In addition, speciation and extinction take place by mutation. The neutral theory pertains in particular to generalists that can adapt to many different niches. The neutral theory was pioneered by Hubbell [129] in 2001, where it was proposed that species at the same trophic level in a community can be viewed as similar in their competitive ability, meaning that biodiversity is randomly established through a random walk, and thus the community is put together in a stochastic fashion.

Much effort has been exerted to establish which of these approaches that dominate in community assembly, and support for each perspective has been found in various studies. It is likely, however, that in many circumstances communities are influenced by both stochastic and deterministic factors, perhaps at different spatial and temporal scales [45], [87].

2.3.1.2 Stochastic vs. deterministic processes in community assembly

The question of the relative importance of stochastic and deterministic processes in community assembly is an area of active research [88], [46]. It is generally accepted that both processes take place in community assembly, but no consensus of when and why one regime takes precedence over the other [219], [229]. Here, deterministic processes refer to selection related to the abiotic environment, and interactions within species, whereas stochastic processes are those that have

an element of unpredictability, such as random growth and death, disturbance events or probabilistic dispersal. One study which attempts to clarify the relative influence of deterministic vs. stochastic events is Stegen et al. [229], which was conducted on subsurface microbial communities in clusters about 30m apart over a timeframe of 10 months, with sampling occurring at irregular intervals from twice weekly to once every two weeks. It was found that phylogenetically similar taxa coexisted more frequently than expected by chance, and that similar taxa have more similar habitat associations. The study concluded that there were spatial and temporal differences in the importance of stochastic and deterministic processes, and that deterministic effects dominate with stochastic processes having a secondary role, but that stochastic processes are important at periods of change in community composition.

2.3.1.3 Predictability of community evolution

A question with close ties to the issue of species sorting and neutral assembly is the extent to which the development of a community can be predicted. Again, there are two different possible scenarios. The first idea is that community development is deterministic and predictable. This means that a certain environment will always generate a community with the same end-point, consisting of the same or similar species from a larger starting set of species, converging through a combination of characteristic environmental pressures. The contrasting theory is that community assembly is inherently divergent, whereby identical environments inoculated with identical communities would generate different end-point communities. There is observational and experimental evidence for both of these scenarios, suggesting that further investigation is needed [191].

2.3.2 Experimental studies of community assembly

Among the experimental studies on microbial ecosystem development is Pagaling *et al.* (2014) [191]. The study looked at whether the community history affected the predictability of the ecosystem evolution, by using freshwater sediment-water microcosms based on Winogradsky columns. Measurements of community function was done by proxy of the redox potential. The experiment tested both convergence of initially different communities and divergence of initially similar communities. The results indicate that the evolution of communities colonising

an unfamiliar habitat is unpredictable, with rare species being strongly selected. On the other hand, allowing communities to colonise familiar habitats yield a more predictable final community, in which rare species are not amplified to the same extent, meaning that the community changes less from its original state at inoculation.

A similar study has looked at community assembly in outdoor sterilised rock pool microcosms [160], with the aim to study whether species sorting or neutral assembly dominates in the early stages of community development. Rock pools were chosen as the environment of choice as they naturally go through annual cycles of desiccation and community re-assembly. Microcosms with different media were placed outside to be inoculated by rain water for a time that was short enough not to become controlled by predation, and were collected after one rain event. The results indicate that the assembly process was dominated, although not exclusively, by neutral assembly, as revealed by the fact that many OTUs were unique in the different microcosms and also detected in the rain collectors. However, rock pools are typically localised, ephemeral habitats, suggesting the need for further systematic studies of the emergence of complex microbial systems in various types of environments.

The laboratory group of Jonathan Adams at Seoul National University have conducted a number of studies relating to community assembly. Kerfahi et al. [147] looked at the colonisation and community assembly of volcanic ash microcosms in a range of climates in Japan. The study was conducted over two years, with DNA 16S rRNA sequencing performed to get a snapshot of the community at 12 and 24 months. The results showed that the bacterial communities were different from other established soils in the area and that many unclassified strains had been picked up. The community assembly was not found to correlate strongly with either temperature, pH or N concentration, with a potential weak correlation to C concentration. The communities were found to be similar in the various microcosm conditions, and dominated by Proteobacteria to about 20-40%, followed by smaller fractions of Chloroflexi, Actinobacteria and Bacteroidetes. They found that most triplicate samples cluster together according to each condition, but with some overlap, and clear separation between the microcosms and the forest soils. They explain this distinction in community composition by the nature of the abiotic environment, the lack of nutrients such as C and N, and the absence of plant roots and their associated community, and conclude that a diverse community is established surprisingly quickly.

Another study by the same group, [219], looked specifically at the role of stochastic vs. deterministic assembly over spatial scales in agricultural soil microcosms on the North China Plain. Here, deterministic assembly is viewed as the premise "Everything is everywhere, but the environment selects" [13]. Stochastsic processes are taken to be those where many species can exist in the same or similar niches as their competitive abilities are closely matched, and relative abundances are determined by chance alterations in populations. Dispersal and arrival are viewed as unpredictable, and previous disturbances can be responsible for population crashes. The difference between these two regimes is determined by applying Mean Nearest Taxon Distance (MNTD), Nearest Taxon Index (NTI), βMNTD, βNTI and zero-sum multinomial (ZSM), where the β methods take into account spatial scales through beta-diversity measures. The study found that stochastic processes are more important on smaller spatial scales, whereas deterministic processes had more influence on larger spatial scales. Furthermore, stochastic processes were found to have a greater impact on the North China Plain, whereas deterministic processes dominate on the Tibetan Plateau. They found that soil pH was the most important factor explaining the community composition of the main samples, but that most of the variation could not be explained by the known environmental or spatial factors, hence calling for more research on stochastic factors. A similar study performed by the same group found a strong link between extreme soil pH and deterministic assembly, while circumneutral pH supported a more stochastic community assembly process [243].

Chase and Myers (2011) [46] presented a framework for disentangling the influences of stochastic and deterministic community assembly for developing site-to-site variation in microbial communities. This is accomplished by adopting a null-model approach where local and regional factors that generate stochasticity are accounted for. They utilise MacArthur's Paradox to illustrate this concept of how the niche, how an organism interacts with its environment, varies across spatial scales. The paradox comes about because differences between species traits are seen as important on local scales, but neutral at larger scales in the theory of island biogeography. In order to resolve this paradox, the true importance of deterministic and stochastic assembly needs to be ascertained. A null model is developed, where niches are assumed to be irrelevant to the patterns of community assembly, taking into account that ecosystems can vary at local and regional scales, giving rise to stochastic effects. Deviations from the null hypothesis indicates a role for deterministic effects, and the amount of deviation is indicative of the strength of the deterministic effects.

A study by Pagaling et al. [192] found that alternative stable states can be generated in identical Winogradsky column microcosms with pond sediment. Identical nutrient-cycling microcosms were set up using natural homogenised pondwater and sediment according to a standard Winogradsky column protocol. For this study, 100 microcosms were set up, and allowed to develop over two or four months before samples were taken. It was found that the communities in the microcosms had diverged into two stable states, dominated by either Firmicutes or Bacteroidetes, and containing distinct sulfate-reducing communities. These divergences are possible due to stochastic factors, whereby feedback and interactions between different species, or species and the environment, can lead to alternative stable states in community structure. Furthermore, it was found that increasing the size of the microcosms decreased the separation between the two stable states. Thus, a smaller system may be more susceptible to stochastic variations and the potential absence of rare taxa at the low-diversity bottleneck, which would have an effect on the subsequent community development. Importantly, this research shows that it is possible to see divergence in microbial communities under identical circumstances, and that these differences may be mediate by stochastic factors.

The effect of a wildfire disturbance on the microbial community assembly process has been investigated [87]. Here, the microbial community was sampled in burned and unburned soils at two time points: 4 and 16 weeks after a wildfire disturbance event. It was found that the community structure and diversity, as well as soil chemistry, was significantly altered for for the burned soils at both 4 and 16 weeks post-fire. A shift in community structure was found between weeks 4 and 16 for both burned and unburned soils. Burned soils was found to have a lower gammadiversity at both time points, by 28% at 4 weeks and 37% at 16 weeks. Burned soils also had a lower alphadiversity than unburned soils, as measured by species richness, with an average of 31% at week 4 and 50% at week 16. The community structure was found to be different for both burned at unburned soils between 4 and 16 weeks, and within each treatment at each of the time points. The relative abundances of each of seven dominant phyla was found to be significantly different for burned and unburned soils at both time points. The burned soils were dominated by Firmicutes at both time points, while in unburned soils Firmicutes were in low relative abundance. Betaproteobacteria were in low relative abundance in burned soils at 4 weeks, but increased in relative abundance to be the second most dominant phylum at 16 weeks post-fire. Alphaproteobacteria was one of the most dominant phyla for both unburned soils at both 4 and 16 weeks, and Actinobacteria were of high relative abundance in

both burned and unburned soils. In terms of community assembly, at 4 weeks burned soils were significantly more stochastic than unburned soils, whereas at 16 weeks, burned soils were significantly less stochastic than unburned soils. This leads the authors to postulate a three-stage model of niche and neutral assembly as a function of time after disturbance, where the first phase sees an increase in the importance of neutral assembly, the second phase is dominated by niche processes, while in the final, third phase, neutral process are again important as the environment becomes less harsh [87].

Evidence has been found to support synergy between species sorting and neutral processes in hypolithic communities, which are found on the underside of rocks, often quartz, in Arctic and Antarctic environments, where the rocks provide shelter from the harsh environment, but at the same time allow for enough light to pass through to mediate photosynthesis [56]. A study by Makhalanyane et al. [172] (2013) found evidence of species sorting in hypolithic communities as the communities were discrete, but that around 90% of organisms in the communities were also found in the surrounding soil, indicating that these communities do not form independently, but are affected by immigration from the surrounding soil.

2.3.3 Differences in community composition depending on rock substrate

Whitman et al. [267] found evidence that different minerals get colonised by different microbial communities in a soil microcosm used to grow the annual grass species *Avena barbata* during a colonisation process of 2.5 months. The three minerals under study, ferrihydrite, kaolinite and quartz, were all found to be colonised by similar but distinct communities of microorganisms, with community composition being dominated by Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes and Acidobacteria. From the same soil inoculum, the minerals were found to host slightly different microbial communities that were also distinct from the surrounding soil inoculum, with kaolinite hosting the communities that differed the most from the rest of the samples, which were more tightly clustered. They attributed the community assembly process most strongly to homogenising selection, meaning that biotic or abiotic factors select for closely related taxa in different soil samples [228]. This supports the idea that the mineral substrate can select for the resident microbial community during colonisation.

2.3.4 Factors influencing community assembly

Community assembly is a fundamental process in microbial ecology whereby an ecosystem is built up from its constituent species. Assembly is often fluid, taking place over timescales from days to years. Furthermore, very rarely are communities static and unchanging, but often perturbations of various kinds, whether environmental or from predation or immigration, continually alters the microbial community, with some species giving room for newer or better adapted strains. In the early stages of community assembly, perturbations or stress can shape the community through altering the survival of certain or all species, leading to a different emerging community than one which did not undergo stress. Factors influencing community assembly can be broadly categorised as dispersal, selection, diversification and drift [189]. These categories was first proposed by Vellend [255] and go beyond the realms of microbial ecology, but are general concepts for community ecology on all scales. Outlined below are the implications and applications of these concepts for microbial community assembly.

2.3.4.1 Dispersal and distribution

One of the key unanswered questions in microbial ecology is the global distribution of microorganisms. One theory states that "everything is everywhere, but the environment selects" [13]. This theory is possibly true because microorganisms are easily dispersed in the atmosphere and through the Earth's hydrological cycle, such that new environments are colonised very quickly. Microorganisms are easily dispersed because of their small size and adaptability, and ability to survive precarious journeys across vast distances, sometimes in spore form. This means that the dispersal of microbes differs from that of larger organisms.

What is well known is that microorganisms as a group are distributed globally in nearly every known environment that is not directly uninhabitable because of certain environmental factors [53]. However, it is not understood whether or not each species of bacteria is readily found, either active or dormant, distributed across the globe [189]. If that were the case, identical environments may host a similar microbial community regardless of how far apart they are on the Earth. This matter is however confounded by other factors that may influence community assembly, such as priority effects and stochastic processes, leading to differing community trajectories even if environmental factors are identical.

2.3.4.2 Selection

Microbial communities are shaped by the environmental conditions in which they reside. In the simplest of terms, if the nutrients required for growth are not present, or some environmental variable is outside the limits of tolerance for survival, the organism will not be able to function in that environment. This is true for all living things. For microorganisms however, the situation is confounded somewhat by the fact that certain organisms may be able to survive inactive for long periods of time, even in environments where they are unable to be active [189]. All microorganisms have inherent parameters within which they can be active, such as ranges of temperature, pH, salinity and pressure, or the presence and absence of certain nutrients or toxins. These selection factors act on the community as a whole, by allowing only those organisms that enter the system and can be active under the present environmental conditions to grow. The microbial community is also shaped by biotic interactions between species in the community, which can be considered part of the overall environmental selection process for individual species in the community [189].

2.3.4.3 Diversification

Another factor influencing microbial community structure is diversification. Here, microbial communities diverge significantly from macroorganisms, in that many microorganisms can undergo rapid evolution. Although there are many unanswered questions regarding these processes, it is thought that, at least under certain circumstances, diversification can take place on similar time scales as other ecological processes. Diversification can take place either through mutations or horizontal gene transfer - in some organisms these processes are thought to be an active strategy in order to adapt to adverse environmental conditions. The implications for a microbial community is that community assembly and structure can be influenced by factors beyond environmental or biotic interactions, such that genetic diversity can originate from processes within the organisms themselves [189].

2.3.4.4 Drift - Stochastic processes

An important factor influencing microbial community assembly is ecological drift, or stochastic changes in the relative abundances of different taxa. In most microbial communities, the relative abundance of most taxa, especially at lower taxonomic rank, is low. This means that many organisms are susceptible to alterations and perturbations of various kinds that can lead to their extinction on a local scale [189]. It is thought that drift is most commonly observed in instances with low alpha diversity and where selection processes are not dominant, and where the overall cell numbers are small [46].

2.3.4.5 Measuring the relative importance of Selection, Dispersal limitation and Drift

A study by Stegen et al. [228] (2013) have proposed a framework whereby the individual contributions of selection, dispersal limitation and drift in community assembly can be determined and linked to the abiotic factors underlying these processes, both measured and unmeasured. From this, they find that selection is responsible for about 33-57% of turnover in community composition, dispersal limitation and drift acting together is responsible for 13-28% of turnover, homogenising dispersal is responsible for 0-21% of turnover, and drift alone is responsible for 22-29% of turnover in the community. Their model was tested on two different geological formations at an unconfined aquifer, and with results suggesting that the 'everything is everywhere paradigm' [13] may not hold true given the importance of dispersal limitation and drift in community assembly, and that this framework has the potential to help distinguish between different factors of community assembly in a variety of environments.

2.3.5 Environmental stress during community assembly

Natural microbial communities always run the risk of coming up against environmental stress or perturbation. Very few environments on the Earth are stagnant or unchanging - most experience some variation in one or several environmental parameters. These parameters can be fluctuations in temperature, air pressure, humidity, rainfall, pH, radiation, predation, salinity etc. Furthermore, the fluctuations can have many different temporal features, such as one-off, irregular

or regular. Even with regular events these can vary in their characteristics and timeframe, such as diurnally, monthly, seasonally, yearly or any other timescale, and can also vary in intensity. Regardless of their nature, fluctuations can act as a stress on the microbial community, resulting in population crashes or varying levels of fatality [157]. Environmental gradients which increase in harshness and thus exert stress on the community in terms of pH, temperature and humidity, have also been found to impact the microbial community [173].

There are currently many unanswered questions relating to the factors influencing community assembly, and the role of environmental stressors or gradients on this process [173], [192]. Depending on the specific composition of a microbial community, it is possible that the community can withstand stress to a greater or lesser degree. A newly established community, or a community in the process of becoming established, typically contain fewer species and may thus be more susceptible to stress, which could have an effect on one or several species, resulting in a community collapse [218], [14], [157]. Equally, young communities may consist mostly of pioneer species that are well adapted to withstand different types of environmental conditions and stress, meaning that the community as a whole is better able to withstand stress [164]. In addition, a community which has yet to establish critical dependencies and complex metabolic pathways between species could also be more resistant to stress, as a fluctuation which causes some limited damage may not result in system collapse. The topic is confounded by the existence of alternative stable states, whereby the community can settle into another composition following either species interaction or environmental disturbance [139], [192]. Alternative stable states can occur through priority effects when there is a variation in the colonisation process, thus creating variety in the possibilities of community structure [45]. Laboratory experiments coupled with field work can elucidate the process, and field studies provide an important way to examine historicity and the effects of long-term exposure [12].

2.3.5.1 Desiccation stress on microorganisms

Desiccation stress is experienced by many microbial communities in a variety of different environments. Some desert communities go through diurnal desiccation cycles, coastal cliff communities may experience drying and rewetting with the tides, and many soils experience periodic droughts and rewetting over periods of weeks to months [258]. Understanding the effects of desiccation is

important as they have a significant and detrimental impact on agricultural soils, leading to crop failure and famine. Desiccation involves three different stages: drying, storage and rewetting. There are four different effects of drying on microorganisms: accumulation of salts and solutes, hyperosmotic stress, metabolism impairment due to low water activity, and damage accumulation if macromolecules lose the aqueous monolayer. During the storage phase, the damage comes from ionising and UV radiation, and reactive oxygen species, as cells cannot perform repair mechanisms in the desiccated state. When the community experiences rewetting, damage can come from hyperosmotic stress or the effect of reactive oxygen species. The survival of a microbial community during desiccation relies on the ability to cope with these stressors [258].

2.3.5.2 Freeze-thaw stress on microorganisms

Natural microbial communities in temperate or frigid climates often experience cycles of freezing and thawing. The frequency and length of the freeze-thaw cycles vary with region, and natural microbial communities are adapted to survive these. Microorganisms in soils experience freezing when the ground cools, but can sometimes still be active, especially beneath a layer of insulating thick snow which keeps temperatures more clement [122]. Freeze-thaw can affect both total biomass as well as shifts in community composition and function, but in certain environments biomass appears relatively unaffected by freeze-thaw. When studying effects over several freeze-thaw cycles, rapid declines have been found to occur either during the first few cycles or only during later cycles. Experimental studies on freeze-thaw cycles in soils show a wide variety of techniques and routines being employed, and calls have been made for more realistic scenarios to be scrutinised in terms of soil profiles and sampling times [122].

2.3.5.3 Experimental studies on environmental stress on the microbial community

The effect of continuous heat stress on a microbial community has been investigated for soils from an arable cropping plot and a permanent grassland in northwestern France [208]. The results show that overall the grassland microbial communities are more resistant to the heat stress, which is expected from a diverse and abundant community. Heat stress was applied continuously throughout the

experiment for a total of 28 days, at temperatures of up to 50°C, on microcosms of sieved soil with natural microbial communities. The microbial biomass was found to be unchanged by the heatstress, whereas the fungal biomass decreased. The microbial community composition and soil function was altered as a result of heat stress, with changes in community structure and function being correlated.

Exerting stress on a plant has been found to have little effect on the microbial community [274]. In a study by Williamson and Wardle [274] (2007) plants were stressed by water limitation and disturbance was caused by defoliation, but the microbial community did not alter appreciably in response to this.

Desiccation stress on methanogens in oxbow lake sediments, an environment which is likely to experience more drying and rewetting cycles in the future, has been studied [60]. One cycle of desiccation and rewetting of the lake sediments was conducted, after which the community was left to grow, and the communities and biomass compared between the communities after and before desiccation. Desiccation here changes the community composition, notably by promoting the growth of *Clostridia*, but the overall gene copy numbers are found to decrease.

The effect of repeated freeze-thaw cycles on a soil microbial community has been investigated mostly using cultured isolates from cold environments. In response to this, a study was set up using laboratory microcosms in order to attain culture-independent results [233]. In this particular study, Himalayan soils and temperate soils that were either adapted (Himalayan) or susceptible (temperate) to freeze-thaw were used. The soil microcosms were subjected to 50-60 diurnal freeze-thaw cycles between -4°C and +4°C, reflecting natural conditions. The microbial community was analysed by 16S rRNA sequencing, and abundance was measured using microscopy. The Himalayan soils showed few changes in response to the freeze-thaw cycles, whereas for the temperate soils a decrease in abundance and respiration was seen, and the community structure was radically altered. In the temperate soils, a successful community re-organisation was observed, leading to survival of further freeze-thaw and the ability to be active at the temperatures above freezing. These results indicate that community response to stress can be determined by its environmental history, however, the two types of microbial communities continued to be distinct in the two soil types.

Another study looked at just one cycle of freeze-thaw, with samples frozen at -20°C for one day before thawing to 10°C and taking measurements for nine days, using a soil microbial community. This study found a shift in the community

make-up after freeze-thaw, but no significant differences in biomass [217].

Sometimes short-lived stress can have dire consequences for a microbial community. An example of this is a study on microbial mesocosms inoculated with natural microbial mats and plankton [193]. The mesocosms were subjected to stress from heightened temperatures and UV radiation, after which the communities altered significantly. The sample richness decreased, while a few surviving taxa dominated the communities post-stress, thus illustrating the potentially drastic changes that can occur in a microbial community subject to environmental stress.

In addition, many studies have been performed that show the impact of stress, environmental filtering or environmental gradients on shaping plant communities and interactions between species, with stress sometimes even driving positive interactions between plant species [61], [163], [244], [36].

2.3.6 Priority effects in community assembly

An unanswered question regarding microbial community ecology is the role of priority effects, or historical contingency, in community assembly [105]. In other words, do species that arrive first to an environment have a higher chance of survival and success than later arrivals? This is an important consideration in line with the argument for stochastic assembly - if species arrive randomly into a system and priority effects are important, then it is likely that replicate conditions would give very different outcomes in terms of community composition. The situation can vary depending on the time scales involved and the rate of immigration and dispersal, with variations in these resulting in different community composition. Priority effects can be either inhibitory, lowering the chances of success of later arrivals, or facilitative, by altering the environment such that later arrivals have a higher chance of survival.

The question of whether priority effects are important is tightly coupled with the consideration of whether the organisms alter the environmental conditions present, such that they are less or more favourable to later arrivals [189], [67]. If pioneer species alter the environment away from its original state to a point where it can proliferate whereas other species struggle to adapt, then priority effects have a significant role to play in community assembly. Vannette and Fukami [251] present three factors which are important for priority effects to be influential,

such that priority effects are stronger when: species have significant resource-use overlap, species are present that are sensitive to environmental alteration, and species are present that notably alter the environment.

2.3.6.1 Experimental studies on priority effects in community assembly

An experiment has studied priority effects among yeasts colonising nectar, by using model microcosms of nectar-inhabiting yeasts [251]. In the natural system, nectar pools in flowers get colonised rapidly by yeasts that arrive in low numbers but grow quickly to significant populations. The experiment was conducted in the lab using microcosms that were pairwise sequentially inoculated with four different yeasts. Four different nectar environments were tested: harsh and rich; harsh and poor; benign and rich; and benign and poor, with the conditions being achieved by altering the amount of carbon source and amino acids. The results consistently showed that the first arrival negatively affected the growth of the second arrival, however, the effects were stronger among some pairs and for some environments than others. In general the benign environments showed stronger priority effects, and stronger effects were also noted for species with a known resource-use overlap.

The same research group has also studied the effect of dispersal on beta diversity, where priority effects are presumed to play a role [252]. In an intriguing experimental set-up, the amount of dispersal into real flowers of a selected species was limited through either netting or bagging a sample set of flowers, and comparing the resulting microbial communities that had been affected by dispersal either only by wind (bagged), or additionally also by bees and insects (caged/netted), and finally also by hummingbirds (no interference). The results indicate that increased dispersal increases beta diversity, contrary to what was previously thought, and the samples that had interactions with hummingbirds differed significantly from the bagged or caged samples. Priority effects can explain the findings, as higher variability in the communities was observed in samples with a high density of organisms, suggesting that species interaction and historical contingency has a role to play.

Priority effects has also been studied on different strains of the same species, *Pseudomonas fluorescens* SBW25 [95]. Three different, distinct genotypes with different morphology and specialist niche were utilised: smooth morph type colonising the liquid, wrinkly-spreader morph type which forms biofilms at the

liquid-air interface, and the fuzzy-spreader morph class which inhabits the bottom of the microcosm, with the three morphs originating from the same ancestral species. When the ancestral strain was added on its own to a new microcosm, the three morphs quickly developed. This was also true if a niche specialist was introduced after some time. If the same niche specialist was introduced shortly before or after the ancestral species, there was a significant reduction in the diversification of the ancestral strain. These results show that the resultant diversity is coupled to the early immigration history and priority effects.

The Fukami lab at Stanford University has conducted many more studies on priority effects, addressing various issues that come into play, such as the impact of historicity on sympatric evolution [282], and the role of priority effects on the infant gut microbiota [227].

The interplay of priority effects and stochastic effects in community assembly has been examined by Chase [45] (2007), by utilising artificial pond mesocosms that experience induced periods of drought. Each mesocosm was seeded with a large amount of producers and invertebrates twice during the 4-year study period, and natural colonisation also took place. The communities in the ponds that did not experience drought were relatively varied in their composition, suggesting that stochastic and priority effects played a role in community assembly. On the other hand, the communities that underwent drought were much more homogeneous in composition, indicating that the harsh environmental disturbance had acted as a niche-selection filter to select for those organisms that could tolerate drought, thus lowering the regional variance and beta-diversity.

Priority effects have also been studied outside the realm of microbiology, for instance using plants, showing that certain plant groups can increase overall above-ground biomass [261], [262].

2.3.6.2 Modelling studies on priority effects in community assembly

Eco-evolutionary buffering has been used to describe the phenomenon of coexistence of species even when priority effects are important and in the absence of immigration from an external species pool [275]. This has been studied by building a model of interspecific interference between two species, where each species has one genotype that is more resistant to interspecific interference. There is a trade-off however, meaning that the resistance comes at the price of decreased

fitness. Because of this, a more common, less resistant strain can be invaded by a rarer, fitter strain, which can subsequently recover in population size on the regional scale. This means that coexistence is possible, such that both the species diversity and intra-specific genetic variation can be maintained even in the face of present priority effects.

Priority effects in community assembly has been modelled by utlising the monopolisation hypothesis, where rapid colonisation of and adaption to a new habitat can result in the earliest colonists monopolising the available resources at the cost of later arrivals [70]. In a study by Urban and De Meester [245], early colonists rise to prominence and inhibit the establishment of later arrivals of an ecologically equivalent species, which has a noticeable effect on the diversity of the resulting community. The study also addresses the interplay between ecological and evolutionary effects in community assembly, through adaption of the resident community in conjunction with the arrival of new colonists.

Several models have been applied to the colonisation of chitin particles in the ocean, and findings corroborated by experimental data [68], [83]. These studies show that bacterial colonisation of chitin particles is characterised by rapid succession and turnover of taxa, and that these changes are impacted by species interaction. The early colonisers alter the environment in favour of the secondary species, which would not have been able to colonise the pristine environment due to their metabolic requirements, thus illustrating the importance of assembly order and priority effects in microbial community assembly.

2.3.6.3 Priority effects in protist communities

Priority effects have found to be important for the community assembly of protists, which are unicellular eukaryotes such as protozoa, algae and slime molds [1]. Warren et al. [260] (2003) have shown that protist communities often assemble to a certain end-point community, regardless of the assembly order and specific species combinations, and that the order of species arrival affects the community trajectory, indicating that priority effects play a role in community assembly. They found that community assembly was influenced by the timely action of invasive species that acted as catalysts, by changing the existing community and then going extinct themselves. It was found that some communities could only be assembled by adding all the constituent species at once, and not through sequential assembly where species were added at different times. They also

found evidence for Humpty-Dumpty communities [200] which cannot be put back together using only the species which the end-point community contains.

Experimental evidence has been provided to suggest that priority effects only matter in the short-term, and that the advantage of early arrival into the system does not last over longer time scales [49]. A study on protists by Clements et al. [49] found that priority effects can work in synergy with temperature to influence community assembly, and that there is thus a link between environmental factors that influence community assembly, and stochastic factors. They found that temperature had a significant effect on the long-term community structure, but that historical contingencies in community assembly also influenced the interaction between temperature and species abundance, providing evidence for an intricate link between stochastic and deterministic assembly factors.

Jiang and Patel [137] (2008) used a protist microcosm experiment to probe the link between priority effects and disturbance during community assembly. They found that in general communities that experienced sequential assembly were more prone to alterations caused by disturbance, where disturbance was induced through sonication, leading to density-independent mortality in the community. This was thought to be because later arrivals were more likely to become extinct or not become established if disturbance occurred in the middle stages of community assembly. It was also found that disturbance weakened the impact of priority effects as competition became less important, and that increasing disturbance led to a lower likelihood of the development of alternative stable states.

2.3.6.4 Priority effects in plant communities

Priority effects are known to be important in plant communities, and have often been studied in the context of grasslands. Priority effects have been found to affect assembly routes and community function, and can be more important than density effects and seeding intensity in affecting total aboveground productivity [103]. A study of the removal of plant functional groups suggest that priority effects may last for a long time after a certain species has been removed from a community [121]. Priority effects in plant communities have also been found to be influenced by environmental variables such as precipitation, and that native species may experience less priority effects than exotic species [113]. Taken together, these studies illustrate the susceptibility of plant communities to priority effects during community assembly.

2.4 Modelling of microbial growth

Efforts have been made to model complex microbial systems in various environments. The subject benefits from a multi-method approach including field studies, laboratory enrichments and modelling. In particular, laboratory experiments may overlook species whose niche is not included in the laboratory simulation of the environment [231, 259]. Modelling enables simplification of an otherwise highly diverse system, especially if functional redundancy can be accounted for [149]. Taken together, laboratory experiments, field studies and modelling efforts enable the advancement of a more complete image of microbial community assembly.

Kettle *et al.* (2014) [149] have modelled the microbial dynamics in the human colon, an area that benefits greatly from modelling due to the invasive nature of clinical trials. The model is based on reducing an otherwise highly complex system to 10 bacterial functional groups (BFG), which are defined from their substrate preferences, metabolites and preferred pH range. Ten types of growth substrates and metabolites are considered, with an internal overlap of six molecules which can function as either substrate or product. A maximum growth rate and yield are defined for each BFG. Each BFG has a preferred pH range for maximum growth, with zero growth outside this. The model takes an emergent behaviour approach, whereby the starting point is 10 strains per BFG, giving 100 strains in total, all of which are defined stochastically within the range of the respective BFG and maintain these values throughout the run of the simulation. Competition between the strains then allows the community to self-organise until it reaches stability. For low-resource environments, strains with high ratios of maximum growth rate to half-saturation constant will dominate, whereas in high-resource environments strains with the maximum growth rate should be most abundant. The model allows for strain succession with adapting the mean trait values of each BFG, giving the expected result that a higher diversity gives an increase in production. The study found that raising the number of strains per BFG led to an increased similarity between runs, due to the strain succession feature, whereas a smaller number of strains per BFG gave a more diverse population. The model was also tested for knock-out of any of the BFGs, with the initial biomass being the same for each group and a pH switch after a certain amount of time. The model generates many similarities with experimental data in terms of the observed changes in phylogenetic groups and metabolites.

In an updated version of the same model, Kettle *et al.* (2018) [148] extended

this concept to a more functional, generalised R package that can be applied to a range of environments or scenarios. In this case, a more automated approach is adopted, whereby dynamical simulations based on ordinary differential equations are generated based on providing a description of the system in question. The functionality of the model and possibility for varying the input parameters was increased, and the system successfully applied to simulating the human colon microbiota and a water column with phytoplankton. In addition, the updated model also contains the possibility of studying the interaction between bacteria and bacteriaphages.

Williams and Lenton (2007) [272] presented a "Flask model" of a microbial community in a flask of liquid with predetermined nutrient influx in a chemostat-like manner. The growth of each organism is determined by food supply and environmental conditions in the flask. The model allows for mutation with low probability during reproduction, generating novel strains. The key features of the model is that it allows evolution of both nutrient uptake and release ratios as well as the organisms' effect on and reaction to the abiotic environment. In contrast to previous work [77], [78], this model separates out the benefits to the individual from those of the environment; the organisms effectively alter the abiotic environment as a free by-product of their metabolism. The flask contains a liquid medium, where some nutrients can be consumed, while others serve only to build up the abiotic environment. Growth occurs when nutrients are converted to biomass and reproduction happens at a specific biomass. Death occurs if the biomass drops below a certain level, and also with a certain low probability at each time step. The simulation was run for a number of scenarios. Running the model without mutation and abiotic constraints gives no recycling, as organisms are not allowed to reuse their own metabolites. Including mutations, still without abiotic constraints, gives a community with single-nutrient consumers that settles into a steady state close to the carrying capacity. Furthermore, introducing abiotic constraints on the growth gives selection for organisms that are adapted to the environment, and environmental changes as a result of mutations cause a time lag in adaption. This can in extreme cases lead to population crashes due to rebel organisms which evolve the abiotic environment away from that which the community is adapted to. The model would benefit from better constraints on adaption, more representative parameter values and more realistic chemistry. It is plausible that the rates of mutation are too high compared with real environments, and the number of organisms included are orders of magnitude smaller than in the environment due to computational constraints.

The Flask model was updated in 2008 to test for whether feedback processes have a stabilising or destabilising effect on the environment [273]. The model incorporates the flask as outlined above, but connects a set of 10 flasks in a ring topology. Each flask is well mixed, with all organisms having the same environmental preferences. However, the flasks can develop in different directions due to imperfect mixing between the flasks. The selection in each flask is exerted by nutrient limitation in good environmental conditions, and by higher-level traits affecting the environment when conditions are bad. This means that communities which improve their environment fare better than those that make their environment less habitable, which leads the whole system to stabilise against external perturbations. This points towards a scenario where the ideas of natural selection can work through a large-self regulated system.

2.5 Microbial weathering

Weathering can be both initiated and accelerated by the actions of microorganisms [247]. The impact of weathering on the Earth system is paramount, affecting, on the larger scales, processes in the Earth's crust, mantle, hydrosphere and atmosphere [16]. At the same time, weathering contributes to industrial applications such as agriculture, architecture, landscape evolution, water and soil quality, nuclear waste processing and mineral distribution [16].

Microbial rock weathering takes place when microbes interact with minerals. Most rock surfaces in clement conditions play host to microorganisms, an important exception being young igneous provinces that have not yet been colonised. Microbes weather rocks through acids, generated as a by-product of their metabolism, which causes dissolution and fragmentation of the rock substrate. This action frees up nutrients to be utilised by the organism itself, or makes them available to other organisms in the ecosystem. In addition, weathering also releases cations that can draw down CO_2 from the atmosphere. Microorganisms on rock surfaces often form complex microhabitats where the local environment is altered to suit the needs of that particular organism. Some species form biofilms consisting of a matrix of extracellular polysaccharides (EPS), DNA and proteins, which provide added protection for the community against environmental changes and stresses [69], [265]. Many of these complex systems are still poorly understood.

Sustainable and healthy soils are fundamental to the past, present and future of humanity on Earth, and understanding the weathering process is central to understanding soil formation under various conditions. Soil systems provide complex habitats for microorganisms and are often composed of various distinct niches [247]. Microbial species that are found directly on the rocks are often different from those that exist in the surrounding soil [42], and the endolithic and epilithic communities are often different [178]. Soils are the result of weathering and mixing with organic matter that takes place over longer timescales than considered in this project, and varies depending on climate, vegetation, topography, parent material and other factors [5].

An active area of research is understanding how specific metabolic functions and genetics are linked to weathering capabilities [247]. One putative function is oxidoreduction reactions, where insoluble electron acceptors in direct contact with the mineral, such as iron, are utilised, with suggested electron transfer via molecules such as quinones, extracellular cysteines and heteropolymers of melanin [247]. This causes weathering by virtue of removing certain building blocks of the rock matrix, hence rendering it unstable. Specifically, feldspar dissolution under abiotic conditions is a process consisting of several steps. Firstly, charge-balancing cations, such as K^+, Na^+ and Ca^{2+}, at the mineral surface are exchanged for protons. This is followed by hydrolysis which forms an activated complex, after which silica and alumina species can be detached from the substrate. In this last step, fresh rock is exposed, which generates new surface area for weathering. Microorganisms can influence this process by altering variables such as temperature, pressure, ionic strength, pH and chelating ligand abundance [21], and hence have a direct influence on rock weathering processes.

Acidolysis is another suggested function for weathering, whereby the microorganisms secrete acids, such as gluconic or carbonic acid, which alter the local pH. In addition, chelation reactions are thought to contribute to weathering through the same mechanisms as acidification, by releasing metal ions from the rock. These processes function by using electron transfer to extract nutrients from the mineral surface. In addition, acids and chelating agents have the ability to break oxygen links. Finally, weathering also occurs through the formation of an imbalance between cation and anion concentrations due to ion chelation. Molecules such as siderophores and catechol derivatives are thought to be directly involved in weathering reactions. Acidification and chelation in weathering reactions can occur both independently and in concert [247].

Nutrient availability *in situ*, such as carbon, nitrogen and phosphorus sources, play a role in the efficiency of weathering. The presence of carbon, in the form of glucose, or nitrogen, in the form of nitrate or ammonium, both enhance the activity or certain microbial species, hence providing more energy into weathering reactions. Nitrogen and phosphorus are required for cell growth and metabolism by many organisms, and their scarcity in the local environment can be limiting to growth [21]. P is required for the generation of ATP, nucleotides and biomass, and organisms typically acquire P from hydrous phosphates or by scavenging it from apatite or apatite inclusions in silicates. Organisms which have adapted strategies to achieve this by changing the environment to facilitate release, such as acid production, ligand chelation or iron reduction, have an advantage in these environments [21]. Rock environments can often be nutrient-poor, either just at the outset after formation before the local environment thanks to colonisation or soil formation, or can remain nutrient poor, such as on bare rock faces. In the first instance, only the pioneer organisms need to be able to survive using only nutrients from the rock or atmosphere, whereas if the environment remains barren and inhospitable, even long-term the microbial community needs to scavenge nutrients from the rock substrate and aeolian influx.

Mineral composition is a major influence that determines the dissolution rates and the readiness with which different rock types weather. Minerals with high silica content tend to be very stable with respect to chemical weathering. Within the silicates, an inverse Bowen reaction series illustrate their readiness for weathering, as the higher the temperature of crystallisation, the less stable the mineral is at the cooler temperatures on the Earth's surface [175]. The Goldich Weathering Sequence describes the order in which minerals tend to dissolve, going from least stable to most stable: olivine, plagioclase, albite, anothoclase $\sim$ microcline, quartz [21, 108]. Minerals with few bonds between silicate-oxygen tetrahedra have weaker structures and hence weather more easily, as do minerals containing iron, magnesium, sodium, potassium and aluminium [175].

The availability of oxygen has an influence on weathering rates. Oxygen levels in the Earth's atmosphere have risen rapidly at several points during its history, between which the levels have remained almost constant. Organisms on the early Earth were exclusively anaerobes. After the initial rise in oxygen levels aerobes came to dominate most surface ecosystems, with anaerobes remaining in select vestiges where oxygen is limited. The way in which oxygen levels affects weathering is two-fold. On the one hand, aerobic metabolisms generate over

ten times more energy than anaerobic metabolisms, which would enable faster weathering rates through a population with high rates of metabolism. However, anaerobic metabolisms contain elements of fermentation which generates high levels of acidity, in turn enhancing abiotic weathering. More information is needed to elucidate the overall effects that oxygen levels have on weathering rates.

Some studies have been conducted on rock weathering by microorganisms under anaerobic conditions, and some anaerobic bacteria are known to have weathering capabilities and being able to reduce Fe(III) and Mn(IV) [128]. A study has been conducted using aquatic mesocosms from an aquifer to look at the weathering action of a natural anoxic community in a laboratory setting [21] (for more detail see Section 2.5.1). This study found that the anaerobic condition seemed to reverse the Goldich Weathering Sequence, implying that microorganisms can radically alter the weathering process of natural rock compared to abiotic processes, specifically under anaerobic conditions.

When undertaking laboratory weathering experiments, it is vital to consider how laboratory weathering rates relate to field weathering rates. Weathering rates have been observed to be up to five times higher in the laboratory compared with field studies [99]. Conversely, modelling this phenomenon based on a rate law for the dissolution of plagioclase in the field indicate that laboratory weathering rates might actually be slower than those in the field. A plausible explanation for this is that secondary mineral precipitation changes the degree of saturation and dissolution rate of the primary mineral [99]. These effect needs to be taken into account when translating laboratory data into predictions for field weathering. There are both benefits and drawbacks of conducting laboratory weathering experiments. The benefits include being able to constrain the system to a few variables that can be controlled and monitored, effectively reducing the system down to a particular sub-set of conditions for scrutiny, but it is important to take into account that this may come at the cost of a loss of realistic conditions or artificial effects not observed in nature.

2.5.1 Key weathering studies

Two studies by Wu *et al.* (2007) and Wu *et al.* (2008) [279], [280] look at the elemental release during the weathering of basalt and granite, respectively, by a single species, *Burkholderia fungorum*. The experiments were conducted over the course of 35 days, with flasks using crushed rock, a minimal weathering medium

with glucose as a carbon source and the microbial inoculum, which were stored in a shaker incubator at 28^{o}C. The samples were analysed for CFU quantities, glucose levels, pH changes, elemental release rates (Al, Ba, Ca, P, F, Fe, K, Mg, P, Si, Sr) and elemental uptake by bacteria (Ca, Fe, K, Mg, Na, P, Sr). It is concluded that pH lowering can contribute to Ca release from trace phases in granite, but less so from silicate minerals. This points to an explanation for the high ratios of Ca/Na in granitic watersheds and suggests that the metabolism of heterotrophic bacteria in the presence of glucose and NH_4 has only a moderate impact on silicate weathering which draws down atmospheric CO_2.

Bennett *et al.* (2001) studied microbial colonisation and mineral weathering in field and laboratory settings, utilising microcosms with sterile mineral chips in permeable polyethylene chambers [21]. In the field, the microcosms were placed in wells or buried in aquifers for 3-12 months, whereas laboratory microcosms were inoculated with environmental samples of sand, peat and groundwater from the field sites. Mineral characterisation was conducted using light microscopy, electron microprobe analysis, trace metal analysis, whole rock analysis, XRF and ICP-OES. Data was collected from biomass measurements, SEM of cell morphology, phospholipid fatty acid analysis, 16S rRNA DGGE and culture enrichment via media selecting for particular metabolisms. The study found no colonisation or weathering of Ontario microcline, oligoclase and plagioclase, moderate diversity and etching of quartz, extensive weathering and colonisation of anorthoclase and South Dakota microcline and extremely heavy colonisation of Columbia River basalt. In addition, no growth was found on olivine, possibly due to lack of a P source, or Ni acting as a biocide. The results do not support the alternative hypothesis that colonisation stems from mineral surface charge differences, whereby microorganisms would be attracted to surfaces with the opposite charge through Coulomb forces. Lastly, it is found that the minerals weather along the expected Goldich weathering sequence [108] under abiotic conditions, whereas the outdoor microcosms follow a reverse weathering sequence compared to the expected Goldich sequence, indicating that certain microorganisms may alter weathering patterns through scavenging.

Glucose metabolism by microbes has been observed to enhance dissolution of bytownite feldspar [263]. A study was conducted using a single species of *Betaproteobacteria* in batch reactors with crushed feldspar in a glucose medium. Experiments were conducted at 5^{o}C, 20^{o}C and 35^{o}C in order to observe temperature effects on weathering rates. The experiments were initially run

for 15 days with measurements taken daily for 3 days and every other day after that. The experiment was subsequently repeated for 3 days with time points taken at 0, 1, 3, 6, 24, 48 and 72h to capture the early gluconate production and feldspar dissolution. Si release rates were used as a proxy for feldspar dissolution. Two types of controls were used: abiotic controls without bacteria and biotic controls with bacteria without a carbon source. These gave a markedly different dissolution response compared to the reactors containing bacteria and a carbon source (glucose), which showed a rapid Si release followed by constant concentration. At 35^oC, no significant gluconic acid accumulation was seen, and no significant difference in weathering compared to the abiotic controls, while at 20^oC, gluconic acid accumulation was seen at the beginning of the experiment, with double the leaching seen in the controls as a result, presumably due to ligand-promoted weathering. At 5^oC significant gluconic acid accumulation was observed, leading to reduction in pH and a 20-fold increase in leaching of Si from mostly proton-promoted leaching. The results suggest an important role for microbial weathering as opposed to abiotic conditions under certain circumstances.

Differences have been found between the weathering rates of basalt and rhyolite in the presence of Cyanobacteria [190]. Basalt and rhyolite are end-member extrusive igneous rocks, whose intrusive equivalents are gabbro and granite. In this study, strains of both fast and slow-growing cyanobacteria were added to the rocks in weathering microcosms, and the elemental leaching and environmental alteration was measured. In general, the presence of the cyanobacteria increased the pH in the microcosms compared to the abiotic controls. The elemental release rates were found to be significantly higher for basalt than for rhyolite, for elements Ca, K, Mg and Si. This difference is attributed to the fact that rhyolite is richer in silica, which has slower weathering rates, meaning that rhyolite overall has smaller abundances of many other bio-essential elements. Overall, the study concludes that cyanobacteria can enhance weathering rates by up to an order of magnitude, which has important implications for the Earth's geological history, potentially providing nutrients for other organisms facilitating CO_2 drawdown from the atmosphere.

Despite these remarkable advances in our understanding of microbial rock weathering, there are areas of knowledge which have not yet been sufficiently covered. There is a lack of systematic studies of microbial weathering under aerobic vs. anaerobic conditions. Furthermore, many studies focus on just a

single species for weathering experiments, hence, understanding how a complex community affects weathering would give insight into how weathering occurs in the natural environment. In addition, there is an important feedback cycle in which weathering is both influenced by and affects community assembly. The aim of this project is to help address some of these gaps in the existing knowledge.

2.5.2 An example of the importance of weathering: The carbonate-silicate cycle

The carbonate-silicate cycle is an important process for regulating the Earth's climate through controlling the level of CO_2 in the atmosphere. CO_2 is an important greenhouse gas with a concentration in the atmosphere of approximately 400 ppm [76]. The cycle describes the different stages of formation and destruction of carbonate and silicate minerals, which links the geosphere, biosphere, atmosphere and hydrosphere. In the atmosphere, CO_2 mixes with H_2O and rains out as carbonic acid, H_2CO_3. Carbonic acid weathers silicate rocks in the following reactions:

$$CaSiO_3 + 2CO_2 + H_2O \longrightarrow Ca^{2+} + 2HCO_3^- + SiO_2 \tag{2.1}$$

$$MgSiO_3 + 2CO_2 + H_2O \longrightarrow Mg^{2+} + 2HCO_3^- + SiO_2 \tag{2.2}$$

These reactions generate the release of cations, such as Ca^{2+} and Mg^{2+}, into water bodies, where carbonates, for instance $CaCO_3$ and $MgCO_3$ are formed by precipitation through interaction with bicarbonate ions (HCO_3^-):

$$Ca^{2+} + 2HCO_3^- \longrightarrow CaCO_3 + 2CO_2 + H_2O \tag{2.3}$$

$$Mg^{2+} + 2HCO_3^- \longrightarrow MgCO_3 + 2CO_2 + H_2O \tag{2.4}$$

The carbonates are subsequently incorporated into rocks through sedimentation. Over geologic timescales, rocks are subducted into the mantle through plate tectonics, where magmatism leads to metamorphosis in which CO_2 is released back into the atmosphere through volcanic processes, such that:

$$CaCO_3 + SiO_2 \longrightarrow CaSiO_3 + CO_2 \tag{2.5}$$

$$MgCO_3 + SiO_2 \longrightarrow MgSiO_3 + CO_2 \tag{2.6}$$

In this way, CO_2 is circulated between the geosphere, biosphere, atmosphere and hydrosphere, through an intricate relationship with silicate and carbonate weathering [25].

Weathering forms part of an important feedback process in the carbonate-silicate cycle linked to the global temperature [23]. Being a greenhouse gas, higher CO_2 concentrations in the atmosphere leads to increased global temperatures and acidity, both of which in turn enable faster weathering. As the reverse is true for a reduction in CO_2 levels, the carbonate-silicate cycle helps regulate temperatures on the Earth [171], by drawing down CO_2 from the atmosphere. Field studies indicate that microorganisms are involved in this process, for instance through cyanobacteria acting as nucleation sites for carbonate minerals in basalt weathering [21]. Laboratory experiments have also shown that microorganisms can enhance dissolution of silicate and aluminium silicate minerals through the generation of organic and inorganic acids, and by producing organic ligands such as gluconate at neutral pH [263], [250].

2.5.3 The Great Oxidation Event - Oxygenation of the atmosphere

A major event in the Earth's history which can be probed in an effort to elucidate biosphere-geosphere interactions is the Great Oxidation Event (GOE). Oxygen levels have increased in several rapid events over geological timescales during the evolution of the Earth. Before the GOE, oxygen concentrations were less than 10^{-5} of present atmospheric level (PAL). At the GOE, around 2.4 Ga, atmospheric concentration of oxygen rose to a few percent of PAL. Current oxygen levels were mostly established around 700 Ma. The first increase, at the GOE, was of substantial proportion and likely had a great effect on the biosphere and geosphere.

For biology, the rise of oxygen presented a novel energy acquisition mechanism which is up to 16 times more efficient at generating ATP than the existing anaerobic metabolisms [216], [41]. Photosynthetic organisms enabled the large increase in oxygen levels through their use of water as an electron donor and oxygen as an electron acceptor to generate energy in the form of ATP. The

ability to utilise this more powerful method of energy acquisition is linked to the emergence of more complex organisms, such as animals [216]. Anaerobic organisms remain on the Earth in anaerobic microenvironments, but aerobic respiration is necessary for most motile organisms larger than $10^{-1} - 10^{0}$m with specialised anatomy [41].

The question of why oxygen arose in concentration at the GOE is intimately linked to the sources and sinks for oxygen. The primary source for oxygen was photosynthetic cyanobacteria, while there are two major sinks for oxygen. The first of these is the oxidation of buried organic matter, both biotically and abiotically. Secondly, oxygen is also consumed in reactions with reduced inorganics from geological processes such as volcanic activity [216], [24]. The total level of oxygen in the atmosphere is determined by the balance between the sources and sinks. For instance, photosynthesis might have been present on the Earth before the GOE, but its effect could have been balanced by consumption of oxygen. Similarly, oxygen levels have stayed nearly constant for long periods of time due to balances between different mechanisms for its generation and consumption.

The ability of different life forms to adapt to oxygenic and anoxic environments has important consequences for rock weathering. Aerobic organisms, which occupy oxygenic surface environments, should be able to weather rocks faster than anaerobic organisms, due to their generally higher rates of metabolism. However, anaerobic fermentation can produce acids which increase abiotic weathering. Anaerobic organisms are pivotal for rock weathering in other anoxic environments, such as subsurface soils, the deep subsurface and water-logged environments. Once the Earth's atmosphere was oxygenated, it is likely that microbial weathering processes overall became more rapid in surface environments where aerobic respiration was the primary mode of energy acquisition.

Some of the first organisms to weather rock at the GOE were aerobic acidophiles weathering terrestrial pyrite. Chromium is used as a proxy for tracking the evolution of O_2 levels in the atmosphere due to the record of Cr isotopes in Precambrian iron formations. Weathering through oxidation of pyrite around the GOE led to rising O_2 concentrations, generating acid rock drainage as evidenced in the Cr record. In this way, analysing the weathering mechanism of early aerobic microorganisms adds to the understanding of the redox evolution of the Earth's crust [155].

In sum, several studies have looked at the effects of the GOE on weathering. However, there is still no systematic study on how this relates to end-member minerals such as granite and gabbro. Part of this project is dedicated to shedding light on this topic and its implications for the evolution of the environments at the Earth's crust.

2.6 Description of rocks under study

In this project, two types of igneous rocks are utilised: granite and gabbro. Granite and gabbro are intrusive igneous rocks with relatively large crystals due to the longer cooling times of the magma underground. The two rock types differ in their silica content, which is determined by fractional crystallisation. This is a process where mafic minerals rich in Fe and Mg crystallise and settle out first, depleting the cooling magma of these minerals, subsequently making the magma increasingly felsic. The point in this process at which freezing occurs determines how mafic or felsic the resultant rock is (that is, it determines the final silica content) [175]. Deliberately choosing a mafic rock type with low silica content (gabbro) and a felsic rock with a higher silica content (granite) enable comparative and contrasting investigation of rock weathering on two globally significant rock substrates. Specifically, a higher silica content generates stability with regard to chemical weathering. Within the silicates, an inverse Bowen reaction series illustrate their readiness for weathering, as the higher the temperature of crystallisation, the less stable the mineral is at the cooler temperatures on the Earth's surface. Minerals with few bonds between silicate-oxygen tetrahedra have weaker structures and hence weather more easily, as do minerals containing iron, magnesium, sodium, potassium and aluminium [175]. In this way, it is hypothesised here that granite would host a community with lower diversity than gabbro, due to a potentially slower release rate of nutrients as it contains a higher amount of silica and lower amounts of many other elements. It is possible, however, that even if the silica in granite weathers slower, other elements could still leach at a faster rate, depending on the minerals in which they are present. Granite and gabbro both present a substrate for a natural community and a global story.

Granite and gabbro are of particular interest as these two rock types together makes up most of the Earth's crust. Granite is the primary constituent of the Earth's continental crust, whereas gabbro makes up the majority of the oceanic

crust, and thus the majority of the surface area available to microorganisms to colonise. The two types of crust have different characteristics, with the granitic crust being thicker at about 30-50km, while the gabbro crust is about 5-10km thick. Although the granite crust is less dense than the gabbro crust, they are both less dense than the underlying mantle, and thus float on top of the mantle through a process known as isostasy. The difference in density of the two crusts means that the gabbroic crust typically sits at lower elevation than the granite crust, hence why the oceans cover these parts. On the early Earth, with little other life and vegetation covering the Earth's surface, microorganisms would have been primarily colonising these two types of rock habitats [179].

2.6.1 Granite

Granite (Figure 2.1) is of interest to this project as it is the primary constituent of the Earth's continental crust and is hence globally relevant and significant to the evolution of the Earth. In addition, granite is known from all geological periods. Granite is felsic, coarse-grained and holocrystalline. The felsic property means that it is rich in elements forming feldspar and quartz, typically the lighter elements silicon, sodium, aluminium, oxygen and potassium. Granite typically contains about 70% silica. Granite weathers physically at exfoliation joints when it expands and fractures, and chemically by carbonic acid and other acids through breaking down of feldspars in hydrolysis. Climate factors also affect granite weathering rates. For granite, studies show that the bacterial community can differ according to the specific mineral inclusion [106, 247]. The specific type of granite used in this study is from Shap, Cumbria, which is characterised by pink Carlsbad-twinned K-feldspar megacrysts and is one of the most common ornamental stones in the UK, for instance used in the plinth of Edinburgh's famous Greyfriar's Bobby statue. Shap granite also contains elevated levels of U, Th, Rb and K [168]. Granite may have been identified on Mars, but is otherwise known exclusively on the Earth [277].

2.6.2 Gabbro

Gabbro (Figure 2.2) is the primary constituent of the Earth's oceanic crust. In contrast to granite, gabbro is a mafic rock with a silica content of approximately 50%. To classify as mafic, the rocks are typically enriched in heavier elements such

as iron and magnesium, with the word mafic being a portmanteau of magnesium and felsic. Gabbro is to some extent thought to be easier to weather than granite and acts as a representative for volcanic rocks in this study [175]. The gabbro used here is from Porthoustock, Lizard Peninsula, Cornwall, which on site weathers to gley and stragnogley soils [82], and from Carrock Fell, Cumbria. Gabbro is abundant in the Earth's crust and is very common on planetary surfaces, making it an important rock type for planetary science.

Figure 2.1 *The Shap granite sample utilised in these experiments prior to being crushed. Sample is circa 15cm across.*

Figure 2.2 *The gabbro sample utilised in these experiments prior to being crushed. Sample is circa 15cm across.*

Chapter 3

Factors controlling the emergence of complexity in microbial communities on igneous rock substrates

Microbial communities are incredibly complex, yet assembled from some of the simplest organisms on Earth. Very seldom do microorganisms exist alone as single species, but rather form complex communities engaged in internal and external ecosystems, enabling the cycling of nutrients and waste products [152]. Currently, there is a good understanding of the behaviour and genetics of many of the most common groups of microorganisms, especially those with direct effects, either positive or detrimental, on human life and society. When it comes to complex environmental microbial communities, there are still large gaps in our knowledge [189]. For instance, there are several theories on how microbial communities become assembled. The metacommunity concept of community assembly proposes, among others, two theories: species sorting and neutral assembly [165]. Species sorting means that the environment selects for the resident community, as species are not equally adapted to present environmental conditions. Neutral assembly sees species as overall similar in their capacities at the same trophic level, meaning that assembly is governed by stochastic factors. Community assembly can be studied for instance during the process of colonising a new, sterile habitat, and there is evidence to suggest that there are different

regimes of community assembly processes over time [87].

An experiment was set up to study community assembly on two different igneous rocks under natural conditions over the course of 18 months, from single species brought in by wind and rain, to a complex, established community. Utilising two different rock types enabled conclusions as to the importance of the environment in selecting for the emerging community, and thus the influence of species sorting on community assembly, or whether stochastic processes appeared to create similar communities across the two rock types. Utilising up to nine replicates allowed analysis of the extent of stochasticity within samples. It was hypothesised that the early community would be dominated by good primary colonists, which would use the rock substrate as a surface only, and hence neutral assembly would dominate early on, but that species sorting would become important over time as the community became more complex and started to utilise the rocks for nutrients as well as adapting to a changing environment. The results indicate the opposite - that species sorting is important early on, as the communities in the two rock types are similar but separate over the first year. Neutral processes appear to become increasingly important as the communities converge after one year. Over time, the environments become more similar as Cyanobacteria come to dominate the community and provide a rich internal carbon source, and pH drops to around neutral for both rock types, from an initial difference of about pH 9 in gabbro and pH 8 in granite. Thus, in this experiment, some of the factors that are important at different timescales in assembling a complex microbial community are observed.

3.1 Introduction

This chapter describes a long-term experiment investigating the colonisation of two different igneous rocks over 18 months, where 16S rDNA sequencing is used to study, month by month, the microbial colonisation of granite and gabbro.

Microbial communities are abundant on the Earth, being present in virtually all habitable environments [268], [53]. Where microorganisms interact with a rock substrate, they often have a profound impact on the geochemical environment, nutrient cycling and soil formation [96]. Through weathering of the rock substrates, microorganisms can affect global climate cycles by the release of cations that lead to the drawdown of CO_2 from the atmosphere [246], [21].

Microorganisms interacting with minerals have far-reaching implications, from biomining and bioremediation to dental plaque and monument decay [206]. Quite apart from rocky environments, ecosystems of microorganisms are present in many environments where their existence is vital to human life as we know it, such as soils and the guts of all living creatures [102]. By understanding how these microbial communities operate, we are better posed to both utilise them for our benefit and tackle them in unfavourable circumstances.

Crucial to understanding microbial communities is the knowledge of how the communities go from single species arriving into an environment to forming a complex ecosystem of resource cycling. Microorganisms, thanks to their small size, ease of dispersal and adaptability to various environmental conditions, are often the first species to colonise a new environment [150], [189], [256]. One such environment is fresh rock, which periodically forms on the Earth through volcanic activity, but also was one of the first types of environments present on the early Earth [169], [264]. Frequently these fresh lava flows are sampled some time after the eruption and the community analysed [176], [145], [94], [240], but no study has previously conducted a rigorous time series of colonisation under controlled circumstances, to see how the community changes over time. The study conducted here was designed to fill that gap, in order to study the emergent complexity of a microbial community being assembled on a rock substrate. Two different rock substrates were tested, in order to elucidate the variation in the emerging microbial community that may be a result of the mineralogy and characteristics of a specific rock substrate and its impact on the geochemical environment [248]. Thus, this study aims to provide new insight into both the influence of time and rock substrate on the emerging microbial community colonising a new environment.

3.2 Unanswered Questions and Hypotheses

Given the current knowledge about microbial colonisation of rocky environments, there are several unanswered questions which this experiment set out to address, broadly on the topic of the factors influencing microbial community assembly, as follows.

- Do different rock types select for different microbial communities? Here, the aim was to test whether there is a significant difference between the

microbial communities that develop in sterilised granite and gabbro over 18 months. It was hypothesised that the communities would initially not be distinct in the two rock types, as the early community would be dominated by generalist, primary colonists that would mainly be utilising the rocks as a surface while gaining most of the nutrients from aeolian input, but that the communities would diverge over time as the community becomes more specialised to the different environments provided by the two rock types.

- How does the emerging microbial community change over time during the colonisation of a new rock substrate? The goal here was to understand how the microbial communities changed over time over the first 18 months of colonisation, and whether the time-dependent changes in the community are influenced by rock type. It was hypothesised that the early community would be dominated by pioneer species that can survive in nutrient-poor environments, notably Proteobacteria that are often found on fresh volcanic lava flows, while the later community would contain a wider diversity of species, notably slower-growing Cyanobacteria, and that these species will be adapted to living in nutrient-poor environments.

- How does the immigration into the system change over the year? In this experiment, this was addressed using fresh microcosms that were deployed at each sampling time and collected with the next sampling time after 1-2 months, and recording whether there is a change in the taxa of microorganisms arriving into the system on a month-by-month basis.

- Do absolute values of biomass of certain taxa change over time? It was hypothesised that early taxa will decrease over time, while secondary species will only become a significant part of the community after the environment is ameliorated by an influx of more nutrients. Notably, slow-growing species such as Cyanobacteria are expected to become prevalent and start affecting the rest of the community only after several months.

- What is the role of species sorting vs. stochasticity in assembling the community? The metacommunity concept [165] outlines four different methods by which an ecological community might be put together, notably species sorting and stochastic or neutral processes, in addition to mass effects view and the patch-dynamics view. The influence of species sorting and neutral assembly was tested by studying colonisation of two different rock substrates under otherwise identical environmental conditions. It was

hypothesised that both neutral processes and species sorting play a role in shaping the community at different scales.

- Can the emerging microbial community composition be linked to changes in different environmental variables, such as pH, elemental leaching etc.? It was hypothesised that the composite effects of different environmental variables would lead to changes in the resident community. pH is expected to alter the community composition by limiting the number of active species at any one time as per their pH range. It was hypothesised that elemental leaching, if observed, would also affect the resident community by providing different nutrients, enabling various metabolic pathways.
- Do changing climate conditions during the year affect the community? It was hypothesised that there would be seasonal variations in the community, linked to harsher temperatures and freezing in the winter months.

3.3 Methods

3.3.1 Methods Overview

This experiment consisted of outdoor microcosms containing crushed rock that were allowed to be colonised by microbial input from the atmosphere. These were placed in a random grid in a box on the roof of the James Clerk Maxwell Building at The University of Edinburgh, Scotland (55.966376° N, 3.2073138° W), from March 2016 until September 2017. During 1.5 years, the microcosms were colonised by microorganisms coming in from the atmosphere, either by wind or by rain. Every 1-2 months, sacrificial samples were removed from the roof and taken into the lab for analysis in terms of pH, elemental leaching, phototroph culturing, cell counts using microscopy, CFU counts on yeast extract agar and DNA extraction and 16S rDNA sequencing. The data were analysed in order to address the question of the factors controlling community assembly on volcanic rocks, and how microbial complexity is established in this environment.

3.3.2 Pilot

A short pilot study was conducted in August 2015 to test the feasibility of the experimental set-up and work out any problems before embarking on the full-scale experiment. For the pilot, triplicate microcosms were prepared with crushed granite and gabbro in 50ml Falcon centrifuge tubes with a hole in the bottom. These were placed in a styrofoam stand and each tube taped down into a hole. The samples were placed on the roof of the James Clerk Maxwell Building for two weeks, and a rock was placed on the styrofoam sample holder in order to ensure it did not blow away. After one week it was evident that something had upset the experiment, as the microcosms lay scattered around the immediate area. It was suspected that this damage had been done by seagulls, who were sometimes seen on the roof, by deliberately picking each microcosm out of its holder. The samples were collected and placed in their original positions, and the whole set-up was wrapped in chicken wire to prevent the samples from being easily removed from the holder. This pilot proved successful as the samples now stayed in place and it was possible to confirm that the flow-through system of liquid worked as there was no overflow of rainwater during heavy showers.

3.3.3 Rock sample selection and characterisation

For this experiment, colonisation of two different igneous rock types, granite and gabbro, was studied. Volcanic rocks are the starting point for much of Earths soil, converted in a process driven in large part by microbial activity. Granite and gabbro were chosen as these are endmember, intrusive, igneous rocks that together make up the bulk of the Earths crust. Granite makes up most of the Earths continental crust, whereas gabbro dominates the oceanic crust. Both parts of the crust float on the mantle, but the granitic crust is thicker, meaning that the gabbroic crust has a lower elevation, and as such, the water in the oceans cover the lower-lying parts [179], [115]. The rock types share some intrinsic similarities, but also have distinct composition and mineralogy. Granite and gabbro are igneous and intrusive, having formed underground, leading to long formation times which gives rise to larger crystals than their extrusive counterparts (rhyolite and basalt, respectively). Granite is classified as felsic (from feldspar and silica), referring to its high silica content of about 70%, making it a relatively acid rock. Gabbro, in contrast, is a mafic (from magnesium and ferric) rock, with a silica content of

around 50%, giving it a higher pH than granite, and is hence also referred to as a basic rock. Thus, while sharing many similarities, there are also distinguishing features of the two rock types that make for a compelling comparison. In addition, granite and gabbro were chosen for historicity, as a previous experiment on the microbial weathering of these two rock types also forms part of this project (Chapter 6).

The rocks used in this study were obtained from different sources. Rocks were ordered from Geology Superstore, Bolton, Lancashire, UK, the gabbro originating from Carrock Fell, Cumbria. Carrock Fell is a well-known gabbro fell near Keswick in the Lake District, which has been mined in the past for tungsten, lead, iron and arsenic. Granite was obtained from Shap, Cumbria, a distinctive pluton formed about 400 million years ago in the Caledonian Orogeny. Shap granite is, thanks to its unusual, large, pink K-feldspar crystals, a common and popular decorative building stone, seen in various well-known monuments such as the plinth of Greyfriars Bobbys statue in Edinburgh and entrance pillars at St Pancras Station, London. Despite these differences in formation and appearance, the distance between the quarries where the Carrock Fell gabbro and the Shap granite originate is only 20 miles. In order to ascertain the chemical and mineralogical make-up of the granite and gabbro used here, samples were analysed by X-ray diffraction (XRD) and X-ray fluorescence (XRF). Analysis was carried out with the help of Nic Odling in the School of Geosciences, The University of Edinburgh. Firstly, the samples were crushed down to a fine powder, using a pestle and mortar. The powder was mounted onto discs that were used to hold the samples in place during analysis.

3.3.3.1 XRD and XRF

XRD uses diffracted X-rays to determine the mineralogy of a sample under study. Briefly, the technique is based on using monochromatic X-rays from a cathode tube, and measuring constructive interference between ingoing and scattered rays. The powder works as a diffraction grating that enables the identification of different minerals due to their characteristic set of values for d, the spacing between diffraction planes, by rotating the sample to measure all instances at which Braggs Law is fulfilled, assuming that a powdered sample has a random orientation of diffraction planes. The final output spectrum can then be matched with one or several minerals that together represent the observed peaks, giving

the total mineralogy of the sample [136]. In this study, XRD was conducted using a Bruker D8 Advance with Sol-X Energy Dispersive dectector at the School of Geosciences, The University of Edinburgh.

XRF relies on fluorescence, whereby atoms in the material are ionised, expelling an electron. Another electron from a higher orbital will take its place, emitting a photon that is characteristic of the energy difference between the two orbitals in question. By determining what atoms are present, the elemental composition of the material can be determined [136]. In this study, XRF was conducted using a Panalytical PW2404 wavelength-dispersive sequential X-ray spectrometer at the School of Geosciences, The University of Edinburgh.

The results from XRF and XRD can be seen in Figures 6.12 and 6.13, Section 6.4.1.

3.3.4 Rock Crushing and Sterilisation

3.3.4.1 Rock crushing

The rock samples were crushed from original kg-sized chunks into smaller pieces with the help of Nic Odling in the School of Geosciences, The University of Edinburgh. The samples were first cut into smaller pieces, before being crushed in a rock crusher to cm-sized pieces. Rocks were further crushed by hand using a steel pestle and mortar, and sieved to a size range of 63μm to 1cm.

3.3.4.2 Rock sterilisation

The crushed rock samples were sterilised by heating in an oven. The rocks were aliquoted into small foil parcels of about 20g each that were left slightly open in order to let moisture escape. The samples were first heated at 90°C for 2h in order to drive off water, after which the foil parcels were sealed and the samples heated at 500°C for 4h.

3.3.5 Microcosm Design

Each microcosm was made up of a 50ml Falcon centrifuge tube. A small hole (2mm) was created in the bottom of each tube using a screwdriver. Firstly, the bottom of the tube was sterilised using ethanol and allowed to dry under sterile conditions in a laminar flow hood. Next, the screwdriver was sterilised using ethanol and then held through a flame. Once cooled, the screwdriver was used to punch a small hole in the bottom of each tube. This hole would allow for the flow-through of rainwater, ensuring that the microcosms would not overflow during heavy or prolonged periods of rain. Each tube was filled with 6.25g of crushed rock (approximately 5ml) of either granite or gabbro. The caps were left on the tubes to keep the samples sterile until they could be placed in their outdoor location. All tubes were also wrapped in sterilised foil to prevent contamination until samples were deployed. Once in place, the lids were removed from the tubes to allow for colonisation.

3.3.6 Control microcosms

Control microcosms were created where immigration was prohibited, as no holes were made and the caps were left on during the course of the experiment. For each time point and rock type, one "dry control" and one "wet control" were created. The dry controls consisted solely of 6.25g of crushed rock, while the wet controls had 6.25g rock and 3ml of sterile water added to it. The idea behind these controls was that they were end-member controls for the environmental conditions that the exposed microcosms would go through - in one instance they were completely dry and in the other they were completely inundated with water. The real wetting and drying cycles that the exposed samples experience were somewhere between these two extremes. The controls were sampled at the same time as the other microcosms, and treated in the same way for analysis.

3.3.7 Box Design

A box for holding the samples in place for the duration of the experiment was designed and built by Scott McLaughlin, School of Physics and Astronomy, The University of Edinburgh. The box had dimensions of 1m x 0.5m x 0.5m, with space for 300 50ml Falcon tubes in a 15 x 20 grid. The box was constructed

such that the tubes were held in place without touching the ground, and with an open bottom to allow rainwater to flow through and drain away. The top of the box was covered by a metal mesh lid with cm-sized holes, in order to prevent disturbance primarily by animals. The lid could easily be removed and put back during sampling. Figure 3.1 shows the layout of the samples in the experimental setting, after 9 time points had been sampled. The covered tubes in the bottom of the image are sterile controls.

Figure 3.1 *Layout of the samples in the experimental setting, after 9 time points had been sampled. The covered tubes in the bottom of the image are sterile controls.*

3.3.8 Random Grid Design

Samples were prepared for a total of 9 samples per rock type per time point, for a total of 12 time points, resulting in a total of 216 samples. Two controls were set up for each rock type per time point, taking the total number of samples to 264. A random grid was chosen to organise samples in the box. Each sample was labelled with a number according to its time point and rock type, from 1 to 216. These were to be distributed over a grid in the middle of the box, avoiding one row at one end of the short edge and two rows at the other end, and one row at either end of the long edge. This meant that the 216 samples were set out over a 12 x 18 grid. A short programme was written in Python, where a list of 216 numbers were allocated random positions using the `random.shuffle()` command from the random package to scramble the list 100 times (see code in Listing 3.1).

The list was then chopped into 12 strings of 18 numbers, and arranged in a grid in a spreadsheet. The control samples were placed in one row of 18 samples at the top of the grid, and in one row of 18 plus one row of 12 samples at the bottom of the grid. This set-up meant that the controls were placed along the edges of the box, while the actual samples were placed at least one row from the edge of the box. This was done in order to minimise edge effects, whereby samples on the edges of the set-up might not be getting the same amount of rainfall or aeolian input as samples in the middle of the grid. The randomisation of the samples as well as sample mixing before analysis (see Section 3.3.11) helped ameliorate the potential influence of edge effects. The final sample placement and legend can be viewed in Appendix A.

Listing 3.1: Random shuffling code for sample grid

```
import matplotlib.pyplot as plt
import numpy as np
import random as rd

L = 216
K =12 #no of rows
M = 18 #no of columns

Vector = [0 for x in range(L)]

for i in range(0,L):
    Vector[i] = i + 1

print Vector

for j in range(0,100):
    rd.shuffle(Vector)

print Vector
```

3.3.9 Experimental Set-Up

The experiment was set up on 10 March 2016, by placing the microcosms in their assigned positions in the box on the roof of the James Clerk Maxwell Building,

The University of Edinburgh, Scotland, UK (55.966376°N, -3.2073138°W). Once all tubes were put in place, the caps were removed from all tubes bar the control tubes. The caps were stored for sterilisation and re-use when sampling.

3.3.10 Experiment Timeline

The experiment ran for a total of 1.5 years, or 551 days, with 12 sampling time points. The sample interval between each time point is indicated, which denotes the duration for which the intermediate control samples, as discussed in Section 3.3.12, were active for.

- Set up: 10/3/16
- Time point 1 (Month 1): 12-15/4/2016 (no intermediates)
- Time point 2 (Month 2): 9-12/5/2016 (4 week intermediates)
- Time point 3 (Month 3): 7-10/6/2016 (4 week intermediates)
- Time point 4 (Month 4): 5-8/7/2016 (4 week intermediates)
- Time point 5 (Month 5): 2-5/8/2016 (4 week intermediates)
- Time point 6 (Month 6): 30/8-2/9/2016 (4 week intermediates)
- Time point 7 (Month 7): 4-7/10/2016 (5 week intermediates)
- Time point 8 (Month 9): 29/11-2/12/2016 (8 week intermediates)
- Time point 9 (Month 11): 24-27/1/2017 (8 week intermediates)
- Time point 10 (Month 13): 21-24/3/2017 (8 week intermediates)
- Time point 11 (Month 15): 15-18/5/2017 (8 week intermediates)
- Time point 12 (Month 18): 12-15/9/17 (4 month intermediates)

The sampling intervals were 4-5 weeks for the first 7 months, and then increased to 8 weeks for the next 5 months, with the final sampling interval being 4 months long. The rationale for this was to increase the total length of the experiment to 18 months instead of 12 months, as few changes were seen in the community in the early month. Thus, extending the overall time offered the possibility to

monitor any potential changes that would occur later. All samples were analysed with these irregularities in mind, and all data normalised to the appropriate timeframe.

3.3.11 Sacrificial Sampling

All sampling was done sacrificially, by removing selected microcosms from the rooftop box and taking them into the laboratory for analysis. Each microcosm was collected by first sealing it with a sterilised cap, and then putting the samples in triplicate into sterile WhirlPak bags (Nasco, USA), in order to avoid contamination through the small drain hole. The samples were removed to the laboratory and immediately prepared for analysis.

Firstly, the samples were mixed together in triplicate. Thus, from the 9 microcosms per rock type per time point, the samples were mixed in triplicate, such that three samples from each time point remained for analysis. For example, the granite samples from the first time point were numbered 1-9. These were mixed in triplicate, such that 1, 2 and 3 were mixed together into one sample, as were samples 4-6 and 7-9. The samples were mixed in a new sterile 50ml Falcon tube, and 3ml of sterile water was added to each composite sample to ease mixing, apart from the first time point, where samples were inundated with water at the time of sampling as a result of sampling during a heavy rainstorm. After mixing the samples together, the tube was shaken by hand for 1 minute, before aliquoting the sample for different analyses. Of the roughly 19g of rock per composite sample, 10g was put aside for DNA extraction, 1g for ICP-OES, 1g for phototroph culturing, 2 x 0.5g for pH measurements, 0.25g for microscopy and 0.25g for plate culturing and enumeration. Samples were aliquoted out using spatulas sterilised by autoclaving, into sterile centrifuge tubes of various sizes (50ml, 15ml, 2ml and 1.5ml).

3.3.11.1 Deviations in sampling at time points 10 and 12

Due to a desire to study stochastic processes in more depth, there were some additional steps added to the sampling procedure in time points 10 and 12. Instead of immediately mixing the nine samples per rock type together into three samples of three, a small, well-mixed aliquot of 0.25g was removed from each of the nine samples, and placed in separate Eppendorf tubes. After this, the main

bulk of each sample was mixed together in triplicate and the rest of the procedure followed exactly as for all other time points, as described above in Section 3.3.11.

These small aliquots for individual DNA extractions were then stored frozen at -20°C until a later date. DNA extractions were performed on these samples using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany), and the samples sent for sequencing analysis in the exact same manner as all other samples, as described in Section 3.3.20.

3.3.12 Intermediate Control Samples

One question being address was whether the organisms arriving into the microcosms were constant or changing during the course of the experiment. In order to study this, intermediate control samples were utilised that were deployed between each sampled time point. These microcosms were constructed in the same way as the original samples 6.25g of crushed rock in a 50ml Falcon tube, with a hole in the bottom to allow for the flow-through of rainwater. When each time point was sampled, three new microcosms per rock type were put out and left to be colonized until the next time point was sampled. For the first six time points, this interim time was 4 weeks, which then increased to 5 weeks, 8 weeks and finally 4 months between time points. When sampled, the intermediate control samples were mixed in triplicate, generating one intermediate control sample per rock type per time point to be used for downstream analysis. The intermediate control samples were treated for analysis in the same way as the original samples.

3.3.13 Phototroph Culturing

The potential presence of phototrophs in the community was of interest as if present, phototrophs provide a carbon source for the rest of the community to use. In order to monitor phototroph growth, flasks for liquid culturing were set up with 1g of rock from each composite sample. To this was added 10ml of BG-11 medium (Figure 3.2), an artificial freshwater medium without a carbon source, known to culture a generic group of algae and cyanobacteria, as a community snapshot was desired [209]. The samples were incubated at ambient laboratory conditions: 21°C and continuous light of approximately 50 μmol/m2/s intensity,

for several months, before being analysed under the microscope, using bright field microscopy on a Leica DM4000B microscope (Leica Camera AG, Germany) at 20X and 100X magnification.

BG-11 recipe

Stock solution 1	**g/250mL**
$NaNO_3$	37.5
K_2HPO_4	1.0
$MgSO_4.7H_2O$	1.875
$CaCl_2.2H_2O$	0.9
Citric acid	0.15
Ammonium iron (III) citrate	0.15
EDTA	0.025
Na_2CO_3	0.5

Stock solution II	**g/1 litre**
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7\ H_2O$	0.222
$Na_2MoO_4.2\ H_2O$	0.039
$CuSO_4.5\ H_2O$	0.079
$Co(NO_3)_2.6\ H_2O$	0.0496

In 1 litre of medium: 10mL stock 1 + 1mL stockII
Adjusted to pH=7.1 then autoclaved

Figure 3.2 *Composition of artificial freshwater medium BG-11, used here for the culturing of phototrophs.*

3.3.14 pH Measurements

Although rocks tend to buffer the pH of solutions, the pH of the rocks was monitored at each time point, using a standard protocol for measuring the pH of soils [40]. Two aliquots of 0.5g rock of each composite sample were placed in two separate 2ml Eppendorf tubes. To this was added 1ml of sterile water. The tubes were closed and shaken by hand for 30s every 5 minutes for 30 minutes. After 30 minutes the tubes were left to stand for 1 hour. After this, the pH was measured using a benchtop pH meter (Omega, UK).

3.3.15 Plate Culturing

The samples were grown on agar plates in order to count and characterise the culturable portion of the community. The process started out with 0.25g of rock in an Eppendorf tube, to which was added 100μl 0.2% yeast extract (1l water, 2g yeast extract, Oxoid, UK). The samples were shaken by hand for 10 seconds per minute for 5 minutes. Agar plates were made using the pour plate method, with 2% agar (agar bacteriological no. 1, Oxoid, UK) and 0.2% yeast extract (Oxoid, UK) (1l water, 2g yeast extract, 20g agar bacteriological no. 1). From each sample, 10ul liquid was plated out in triplicate. Serial dilutions down to x10^8 dilution were conducted as appropriate to each time point. Plates were incubated at 21°C for 1 week, and were counted after 1 day, 5 days and 7 days, in order to distinguish the fast-growing, opportunistic portion of the community.

3.3.16 Cell Count Microscopy

Cell counts were conducted using a Leica DM4000B microscope (Leica Camera AG, Germany). The samples were prepared using an amalgamation of various protocols designed to wash cells off rocks. The first step was to add 1ml of water and 100ul of 0.1M Na4PPi (final concentration 0.01M Na4PPi), which is a dispersant used for washing cells off rocks [146], to 0.25g rocks in an Eppendorf tube. The tubes were vortexed briefly to mix them together. After this, the tubes were sonicated for 5 minutes using a sonicator (Fisherbrand^{TM} FB 15050, Thermo Fisher, UK). Next, the samples were spun down in a benchtop microcentrifuge (Thermo IEC Micro CL 17 centrifuge, Thermo Fisher Scientific, UK) at 600g for 5 minutes. An aliquot of 200μl was taken out and stained using 10μl of x20 SYBR Gold DNA binding dye (Life Technologies, UK) and incubated for 15 minutes in the dark. The cells were fixed on a 0.2μm polycarbonate filter paper (Merck Millipore, UK) by washing the 210ul of suspension using a vacuum pump (Fisher Scientific, UK). The filter paper was placed on a glass slide and a droplet of antifade was added to the top of the filter before adding a cover slip on top. The cells were imaged using a Leica DM4000B microscope at x100 magnification under blue light from fluorescence prism I3. Cells were counted in 150-200 fields of view on a 100 x 100μm grid (200 fields of view for the first few time points when cell density was low, and 150 fields of view for the final time points).

3.3.17 qPCR

A proxy for biomass was obtained by performing qPCR (quantitative polymerase chain reaction) on the DNA that was extracted from each sample, as is described in Section 3.3.20.1. qPCR, alternatively known as real-time polymerase chain reaction, or RT-PCR, allows monitoring of the DNA amplification in real-time, as compared to normal PCR where the quantification is performed solely at the end. For qPCR, a fluorescent dye is added to the DNA before amplification, such that the increase in copy numbers can be continuously monitored in a thermal cycler with a fluorescence sensor [35], [167]. The thermal cycling copies the DNA strands by repeated three-step cycles of heating to different temperatures, at which certain reactions will occur in the solution with the DNA strands. The solution contains primers, which are intended to bind to specific parts of the DNA, and DNA Taq polymerase, an enzyme which helps to assemble a new complimentary strand of DNA from single nucleotides [212]. In the first step of PCR, the DNA is melted, such that the two strands are separated. The temperature is then lowered in the second step, allowing the primers to bind to each DNA strand. For the third step, the temperature again rises to about half-way between the two other steps, and here the polymerase builds the complimentary strand from the free nucleotides. For each repeated cycle, the amount of DNA is copied and exponentially amplified. qPCR follows this process, but with the added feature that, through the use of standards and fluorescence, the total amount of DNA copies in each sample is quantified [17].

qPCR analysis was performed by Lisa Wink at The Medical University of Graz, using primer pair 331F (TCCTACGGGAGGCAGCAGT) forward and 797R (GGACTACCAGGGTATCTAATCCTGTT) reverse [188] in order to amplify the 16S rRNA gene, using the same equipment as and according to a protocol by Pausan et al. [197], using a fixed amount of 4μl of DNA template. These primers are a standard primer pair for qPCR of the 16S gene. These results give a crude quantification of the amount of biomass in each sample, which can then be monitored over time, however, it is not an exact method as the number of DNA copies per cell is not known, and with current techniques it is not possible to know this for each member of the community, and even an average is difficult to estimate [198]. Here, as the analysis is performed on a community, it is presumed that the relative quantification is correct, such that samples can be compared within the experiment, although the absolute values are unknown.

3.3.18 Measurements of Elemental Leaching

In order to measure the elemental leaching from the rocks, the samples were prepared for ICP-OES and ICP-MS analysis. As the rock microcosms were a flow-through system, it was not possible to measure the leaching rates *in situ*. Instead, a protocol was deviced to measure the leachability of the samples in the laboratory. To this end, 1g of each composite sample had 5ml of sterile water added to it, and was left to soak for 1 week, after which the supernatant liquid (4ml) was removed using a syringe, and filtered through a 0.22μm filter, and 100μl of 38% HNO3 added to each sample, to a final concentration of 1.5% HNO3. The samples were analysed with the assistance of Lorna Eades at the School of Chemistry, The University of Edinburgh, Scotland, UK.

ICP-OES and ICP-MS are two similar spectroscopy techniques utilising the properties of inductively coupled plasma (ICP) in order to detect various chemical elements. Argon gas is used to create a plasma at 7000K, by ionisation in an electric field. For ICP-OES (optical or atomic emission spectroscopy), the sample is converted into mist through a nebulariser, and the ions and electrons in the plasma causes the molecules in the sample to separate into the constituent atoms. These atoms are repeatedly turned into ions by losing their electrons and then recombining, giving rise to radiation as the electrons excite and de-excite. The radiation signature is unique for each element, and thus the presence and quantity of each element in a sample can be determined. ICP-MS instead relies on mass spectroscopy (MS), although the principle of ionising argon gas into a plasma is the same, before the sample enters a mass spectrometer whereby the ions are separated according to the mass-to-charge ratio. Both ICP-OES and ICP-MS relies on using standards of known concentration in order to benchmark the concentration of the various elements in each sample [239], [85], [126].

3.3.19 Carbon source utilisation using Biolog plates

For one time point, after the experiment had run for 6 months, the ability of the communities to utilise different carbon sources was assessed, with the aid of a Biolog GP2 MicroPlate^{TM} (Biolog, Hayward, CA, USA). Firstly, a minimal medium, M9, without a carbon source was made up (Tables 3.1 and 3.2). The M9 medium contains many essential trace elements and nutrients to culture environmental strains.

Table 3.1 *M9 recipe overview*

Reagent	Amount
M9 salts	200ml
1M $MgSO_4$	2ml
1M $CaCl_2$	100μl
$FeCl_3$ (anhydrous) at 252mg/l	10ml
MQ Water	770ml
Total volume	1l

Table 3.2 *M9 salts composition (x5)*

Reagent	Amount
Na_2HPO_4-$7H_2O$	64g
KH_2PO_4	15g
NaCl	2.5g
NH_4Cl	5.0g
MQ Water	make up to 1l
Total volume	1l

From each of the triplicate samples of each rock type at this time point, 1g of rock was taken out and mixed together, giving a total of 3g rock mixture. To this was added 12ml of M9 medium, and the tubes were shaken vigorously at 5min intervals for 30mins, and then left to stand for another 30mins. The liquid was poured into a sterlie Petri dish, and 100μl of liquid was added to each well of the Biolog microplate using a multichannel pipette. The plates were incubated at 21°C for 7 days before results were collected, by counting the number of purple wells, and thus assessing whether the colourimetric assay indicated growth of the organisms.

Biolog plates provide an easy and quick way to measure the abilities of an organism or community to utilise various different carbon sources. The plates work through a colourimetric assay, where the wells change colour if the organisms are able to metabolise that particular carbon source [28]. In contrast to many other assays designed to measure metabolism, Biolog plates do not rely on the production of metabolic byproducts, but on the reduction of tetrazolium as a response to metabolism or respiration [185]. In the presence of cellular respiration, the white salt tetrasodium is converted to a reddish TPF (1,3,5-triphenylformazan), and this colour change can be observed macroscopically with the naked eye, thus allowing for identification of which carbon sources the organisms can metabolise and thus indicates their functional capabilities.

3.3.20 Community Composition

The community composition was analysed using a suite of molecular techniques. DNA was extracted from the bulk samples, after which the samples were sent for PCR and 16S rRNA sequencing at RTL Genomics, Lubbock, TX, USA.

3.3.20.1 DNA Extractions

DNA extractions were carried out using the MoBio PowerMax Soil DNA Isolation Kit (MiBio, Carlsbad, CA, USA), according to protocol. For each extraction, 10g of rock sample from a composite sample (as described in Section 3.3.11) was used as the starting material. In the final step, 5ml of buffer C6 was used to elute the DNA. Samples were sequenced using the Illumina MiSeq platform (RTL Genomics, Lubbock, TX, USA).

The DNA extraction kit functions through an intricate set of steps that help extract the DNA from cells, and elute them in a buffer for downstream analyses. The first step involves adding a buffer and the soil sample to a tube, which contains small beads that help lyse the cells. The solution has a triple purpose: to disperse the soil particles, to degrade the humic acids present in the soil that inhibit amplification, and to protect the DNA from degrading. The tubes are vortexed in order to begin breaking up the cells. In the second step, another solution containing SDS and other disruption agents is added, which helps to further cell lysis, and helps break down the lipids and fatty acids that constitute the cell membranes, as the tubes are vortexed for 10mins. Next, the tubes are centrifuged, such that the soil and beads are pelleted at the bottom of the tube. The supernatant is removed and placed into another tube, and another solution is added to it, which has the purpose of precipitating organic and inorganic contaminating material other than DNA. The tubes are again centrifuged and the supernatant transferred to a new tube. A new solution is added which is high in salt concentration. The salt has the purpose of allowing DNA to bind to a silica membrane on a spin filter, while any residual humic substances, proteins or cell debris pass through. The solution is added to the spin filter in three aliquots and centrifuged between each aliquot, ensuring that the DNA is bound to the membrane at each step, while after centrifuging the unwanted solution is discarded before new solution is allowed to bind. With the DNA still bound to the silica membrane in the spin filter, an ethanol wash solution is added

and centrifuged out, such that any final residual unwanted organic or inorganic substances are washed away from the DNA. The spin filter is centrifuged twice to ensure that any remaining ethanol is washed off the DNA, as it would otherwise interfere with downstream analyses. Lastly, the DNA is eluted using 10mM Tris, which is added to the spin filter, and the DNA binds to the solution as it is washed through the membrane during a final period of centrifugation. The DNA is stored in the Tris buffer at $-20°$C until PCR amplification and DNA sequencing [203].

3.3.20.1.1 Pooling of samples for 18S rRNA analysis. For Month 6, the samples for 18S rRNA analysis of the eukaryotic community were analysed in triplicate in the same way as the 16S rDNA analysis, while for all other time points the triplicates for each rock type were pooled after DNA extraction, such that only one sample was analysed per time point and rock type.

3.3.20.2 PCR

PCR is the exponential amplification of DNA through a polymerase chain reaction, as described in Section 3.3.17.

3.3.20.2.1 In-house PCR for quality check PCR was initially carried out in the laboratory in order to determine whether the DNA extraction had been successful. The PCR was conducted using primers 27F and 1389R (Table 3.3), targeting the hypervariable region of the 16S gene [93], [125], [74]. Primers are short strands of DNA of typically around 20 nucleotide bases that help target specific regions of the DNA, a technique based on the cell's innate functions for replication *in vivo*. The specific primers are designed to match the beginning and end of the DNA region under consideration, thus a forward and a reverse primer are used. These primers bind each bind to one of the DNA strands, and they direct the replication towards each other. This means that when the DNA is sequenced, two parts are generated, which later need to be stitched together in the data analysis process. Primer pairs need to have similar annealing temperatures such that the process occurs simultaneously for both strands in PCR [212]. A 25μl reaction per sample was prepared using the GoTaq G2 Colourless Master Mix according to the proportions presented in Table 3.4. PCR runs were performed on a thermocycler (G-Storm GS1, Gene Technologies Ltd., Braintree, UK) according to the protocol outlined in Table 3.5. The PCR products were run on a 1.2%

agarose gel stained with SYBR Gold to confirm the presence of extracted DNA in all samples before sequencing.

Table 3.3 *Primers used for checking DNA extraction yield*

Type	Gene	Forward name	Forward sequence	Reverse name	Reverse sequence
Bacterial	16S	27F	AGAGTTTGATCMTGGCTCAG	1389R	ACGGGCGGTGTGTACAAG

Table 3.4 *Reagents used for in-house PCR*

Reagent	Amount
Forward primer (0.01mM stock)	1.0μl
Reverse primer (0.01mM stock)	1.0μl
Master Mix	12.5μl
MQ	9.5μl
Sample	1.0μl
Total volume	25μl

Table 3.5 *PCR program for in-house PCR*

Step	Temperature	Duration	Number of repeats
Initial denaturation	94°C	4 min	
Denaturation	94°C	0.5 min	35 times
Annealing	54°C	0.5 min	
Elongation	72°C	0.5 min	
Final elongation	72°C	5 min	
Cooling	4°C	10 min then hold	

3.3.20.2.2 External PCR for sequencing preparation In order to prepare the samples for sequencing, PCR was performed at RTL Genomics, Lubbock, TX, USA, according to a two-step process. Samples were prepared with (5-3) the Illumina i5 sequencing primer (TCGTCGGCAGCGTCAGATGTG-TATAAGAGACAG) and the gene specific (16S or 18S) primer as the forward primer, and for the reverse primer with (5'-3') the Illumina i7 sequencing primer (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG) and the gene specific (16S or 18S) primer. For bacterial 16S rRNA amplification, primers 28F (GAGTTTGATCNTGGCTCAG) and 388R (TGCTGCCTCCCGTAGGAGT) were used. For archaea, archaeal primers 517F (GCYTAAAGSRNCCGTAGC) and 909R (TTTCAGYCTTGCGRCCGTAC) were used. For eukaryotes, universal primers MSTAReuk CCAGCASCYGCGGTAATTCC and ACTTTCGTTCTTGATYRA were used to amplify the 18S rRNA gene (Table 3.6).

Table 3.6 *Primers used for Illumina MiSeq*

Type	Gene	Forward name	Forward sequence	Reverse name	Reverse sequence
Bacterial	16S	28F	GAGTTTGATCNTGGCTCAG	388R	TGCTGCCTCCCGTAGGAGT
Archaeal	16S	517F	GCYTAAAGSRNCCGTAGC	909R	TTTCAGYCTTGCGRCCGTAC
Eukaryotic	18S	V4F	CCAGCASCYGCGGTAATTCC	V4R	ACTTTCGTTCTTGATYRA

PCR was performed on a ABI Veriti thermocyclers (Applied Biosytems, Carlsbad, California) according to the reaction concentrations in Table 3.7 and cycling conditions in Table 3.8.

Table 3.7 *Reagents used for external PCR for sequencing*

Reagent	Amount
Forward primer (5μM stock)	1.0μl
Reverse primer (5μM stock)	1.0μl
Qiagen HotStar Taq Master Mix	22μl
Sample	1.0μl
Total volume	25μl

Table 3.8 *PCR program for external PCR for sequencing*

Step	Temperature	Duration	Number of repeats
Initial denaturation	95°C	5 min	
Denaturation	94°C	30s	35 times
Annealing	54°C	40s	
Elongation	72°C	60s	
Final elongation	72°C	10 min	
Cooling	4°C	Hold	

3.3.20.3 16S and 18S rRNA Sequencing

Sequencing was performed using Illumina MiSeq at RTL Genomics, Lubbock, TX, USA. Samples were sent for sequencing within a few months of each time point, and sequenced for bacterial and archaeal 16S rRNA and eukaryotic 18S rRNA. Paired-end sequencing with a read length of 250bp was conducted.

Illumina MiSeq is a high-throughput DNA sequencing technology by which each base in the eluted and amplified DNA strand is identified as either adenine (A), thymine (T), cytosine (C) or guanine (G). High-throughput sequencing means that many sequences are run in parallel, using reversible dye-terminators and polymerases. The DNA and primers are bound to a surface and amplified using polymerase in a similar way to how PCR is performed (Section 3.3.17), in order to create DNA clusters of copied DNA. Loose nucleotides that have

been fluorescently labelled are added, and bind to the DNA strand. As binding occurs, a fluorescence signal specific to each nucleotide base is emitted, which is measured by a camera. Each base in the DNA strand is sequenced and identified in turn, the camera capturing each successive nucleotide binding such that the strand can be interpreted from its reverse fluorescent signal. Each base is given a quality score (Phred score), which indicates the likelihood that the base has been correctly identified. Thus, the whole DNA sequence of the particular targeted gene or section is identified. When part of the 16S gene is targeted, as in these experiments, the sequencing results help the identification of each organism present in the sample, as the 16S gene is very well conserved within a species but hypervariable between species, and allows species or genus identification by comparing the DNA sequence with a reference library [133], [134], [132], [93], [43].

3.3.21 Functional Genes

Extracted DNA was also tested for nitrogen fixation genes using primers mnifHF (TGYGAYCCNAARGCNGA) and mnifHR (ADNGCCATCATYTCNCC) at RTL Genomics, Lubbock, TX, USA, however, PCR amplification was unsuccessful and thus this was not pursued further.

A rudimentary inferred functional gene analysis was carried out using Picrust [161]. This software infers the functional profile of a microbial community using a marker gene survey from a 16S OTU table. The closed-reference OTU table was generated using GreenGenes 13.5 [73]. Here, the online Galaxy platform on the Langille Lab (v1.1.1) server was used. To perform the analysis, the OTU table for all samples at all time points was provided, and the program normalises the OTU table and then predicts the functions for the metagenome.

3.3.22 Sampling and analysis of an established soil community

In order to compare the microbial communities, particularly the end-point communities, with an established soil community, soil samples were collected in conjunction with time point 12 on days without precipitation in September 2017. These samples were collected from areas of volcanic bedrock in close proximity of the experimental location. As there are no truly pristine igneous soils in the UK

due to glaciation, the samples were collected from scree slopes on igneous bedrock in Edinburgh. Scree slopes were chosen to mimic the experimental conditions, as they consist of crushed rock with little vegetation influence, and consist mostly of the igneous rock that the overlying outcrops are made of. Two sample sites were chosen both at Arthur's Seat (55.944584°N, -3.165400°W) and Blackford Hill (55.920128°N, -3.190191°W), resulting in a total of four sample sites. The Blackford Hill samples were collected on andesite/trachytic-rock substrate and the Arthur's Seat samples were collected on basalt and olivine anacline-microgabbro.

At each site, triplicate samples were collected in a sterile manner using 50ml centrifuge tubes and returned immediately to the lab. One set of samples was stored overnight at +4°C, while the other samples were collected the next day, immediately after which a DNA extraction was performed on all samples. DNA extraction was performed using the MoBio PowerMax Soil DNA Isolation Kit (Carlsbad, CA, USA) and the sequencing analysis was conducted as described above in Section 3.3.20.

3.4 Data Processing

Data from all analyses were processed according to standard methods. Specifically, sequencing data were treated using a Qiime pipeline in order to be easily interpreted, as can be seen in Appendix B.

3.4.1 Analysis of 16S rRNA data

The sequences were analysed in Qiime [38], following a standard pipeline (Appendix B) starting with the FASTA files. The sequences were converted to FASTQ files and trimmed of barcodes, and sequences with a Phred score of Q20 or above were retained. Chimeras were removed using USEARCH61 [81] in accordance with the 97% Silva 119 release [204], [281], [107] and OTUs were defined by open reference OTU picking using the same database, while the remaining sequences were clustered de novo using UCLUST [81]. The resulting tree was used for diversity analyses to a rarefaction depth of 9300 sequences, which was slightly below the sample with the lowest number of sequences. The resulting community composition was displayed in stacked bar charts. Beta diversity analyses were displayed in PCoA plots [39].

3.4.1.1 Analysis of generalists

In order to estimate the number of generalists in the samples, we counted the genera that were shared among all samples from all time points, by removing all genera that had an abundance of zero (0) in any sample [87].

3.4.2 Analysis of 18S rRNA data

18S data for eukaryotes was analysed in Qiime [38], following the standard pipeline for 18s data, which varies slightly from the 16S pipeline which can be found in Appendix B. Specifically, the OTU picking, assigning OTUs to taxonomy, and separating OTU tables according to the domain all need to be conducted against a database containing 18S data. Here, the Silva 108 database was used [204], [281], [107].

3.5 Results

This experiment set out to probe the factors governing community assembly of a microbial community on a fresh rock substrate, to study whether different rock types would influence the emerging resident microbial community, and how the community would change over time over the first 18 months of inoculation.

3.5.1 Environmental Results

Over the course of the 18 months of the experiment, the environment changes as measured by variables that were monitored.

3.5.1.1 pH

The pH of the rock substrate was measured at each time point as described in Section 3.3.14. The rocks initially have slightly different pH values, with gabbro at about 9.5 and granite slightly more acidic at around 8.0. During the course of the experiment, the pH drops in both rock types, with final values of just above 7.0 for gabbro and just below 7.0 for granite, as seen in Figure 3.3. The pH

decreases are statistically significant at the 0.05 confidence level between the first and the last time point in both granite (t-test, $p=4.73 \cdot 10^{-5}$) and gabbro (t-test, $p=5.52 \cdot 10^{-11}$) The findings are consistent with the elemental composition of granite being more acidic than gabbro [101]. It is evident that the flow-through system prevents a net build-up of acid in the system, as would likely be the case with a closed microcosm, as found in Chapter 6.

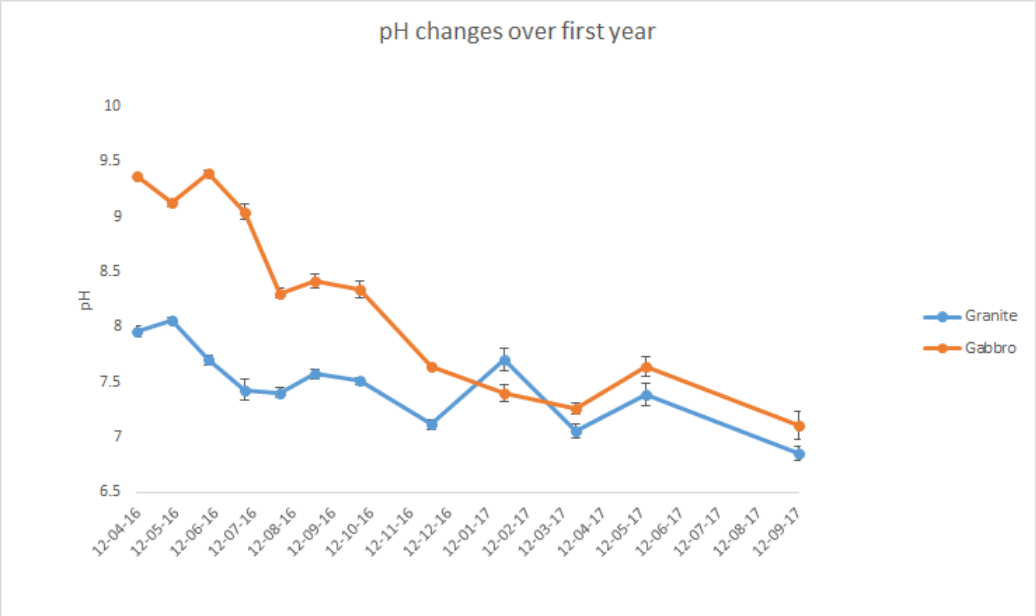


Figure 3.3 *pH for the colonisation experiment over 18 months. Error bars are standard error (n=6).*

3.5.1.2 Elemental Leaching - ICP-MS and ICP-OES

Elemental leaching for this experiment was measured using a test of "leachability" of the rocks by soaking them in fresh, sterile water over 1 week, as opposed to the more common method of measuring the elemental composition in the supernatant, as no supernatant was available due to the set-up of the microcosms. A summary of the differences in leaching patterns are available in Figure 3.4. ICP tests were only conducted on samples from Months 1, 6, 7, 12 and 18. Results are found in Figures 3.5 (ICP-OES) and 3.6 (ICP-MS).

Increased leaching is seen for both rock types for Ni, Zn, Mn, Co and Cu, while leaching decreases for both rock types for Ti, Na and Si. The elements that have higher leaching rates at the end of the experiment would be more freely available at the end, whereas the elements that leach slower at the end of the experiment would have been more prevalent during the early months.

	Decrease	Increase
Granite	Ti, Na, K, Ca, Mg, S	Mn, Ni, Zn, Fe, Cu
Gabbro	Ti, Na, Al, Fe	Mn, Ni, Zn

Figure 3.4 *Summary of ICP results of elemental leaching changes between 1 and 18 months.*

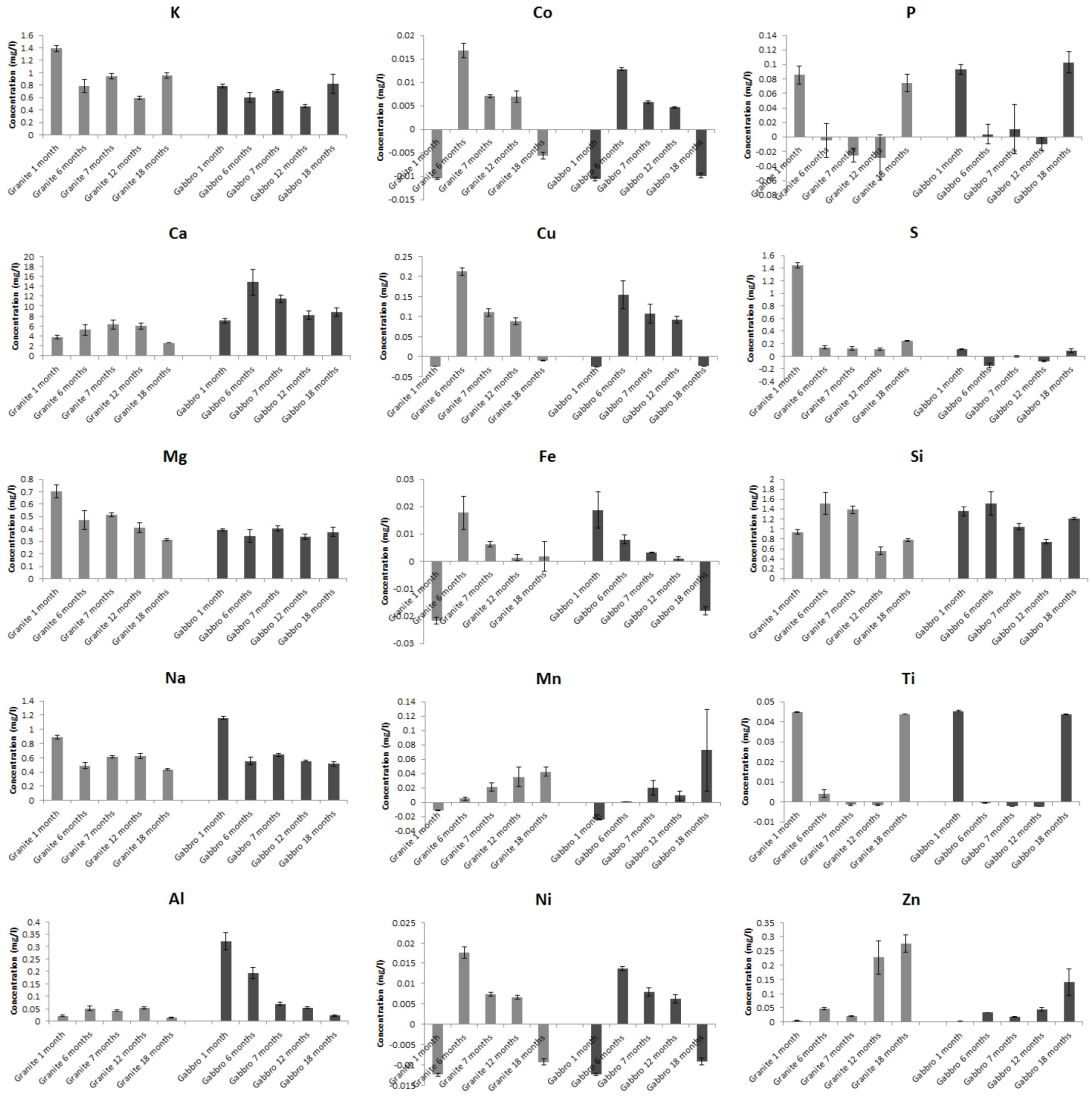


Figure 3.5 *ICP-OES data of elemental leaching changes over 18 months, as measured at time points 1, 6, 7, 10 and 12 (Months 1, 6, 7, 12 and 18). The graphs show, L-R: Granite Months 1, 6, 7, 12, 18 (grey), Gabbro Months 1, 6, 7, 12, 18 (black).*

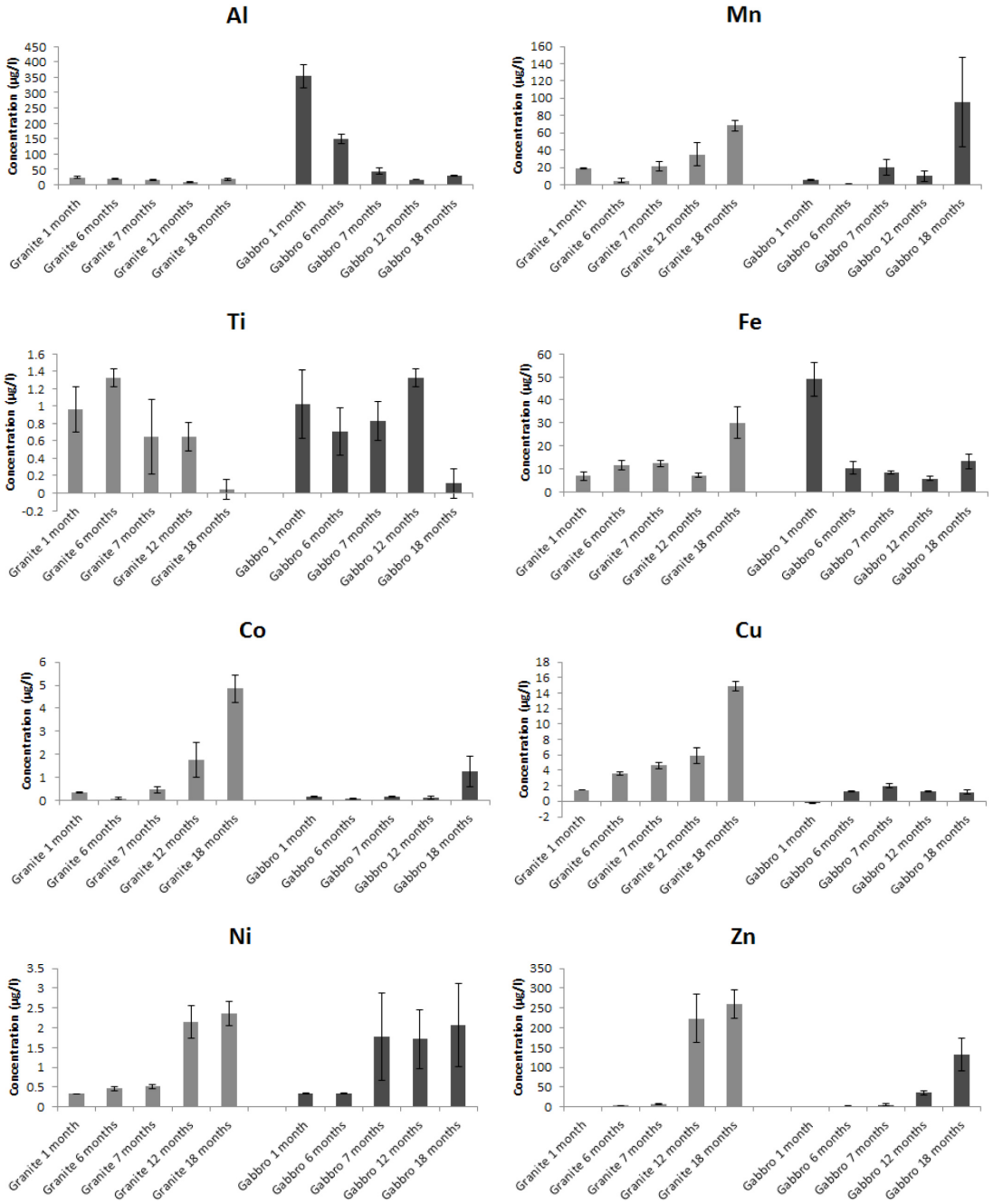


Figure 3.6 *ICP-MS data of elemental leaching changes over 18 months, as measured at time points 1, 6, 7, 10 and 12 (Months 1, 6, 7, 12 and 18).*

Fe is an important element involved in many metabolic pathways. It is found that the concentration of Fe in solution increases for granite between months 1 and 18, but decreases over the same time period for gabbro. The concentration at the end of the experiment for gabbro is similar to that of granite at the start.

ICP-OES	ttest rock type TP1	ttest rock type TP12	ttest time granite	ttest time gabbro
K	0.000669	0.425132	0.002886	0.843386
Ca	0.005703	0.001712	0.032734	0.137354
Mg	0.004522	0.186926	0.001952	0.732609
Na	0.001166	0.078657	8.46E-05	5.77E-05
Al	0.000973	0.084563	0.185628	0.000981
Co	0.930602	0.007962	0.002457	0.371968
Cu	0.209986	5.53E-05	5.56E-05	0.056585
Fe	0.003861	0.023928	0.013227	0.00561
Mn	0.000128	0.625429	0.001057	0.165521
Ni	0.941964	0.902615	0.017861	0.022142
P	0.611826	0.207875	0.565488	0.570402
S	5.48E-06	0.005431	7.27E-06	0.419314
Si	0.017408	0.000504	0.05349	0.211768
Ti	0.194787	0.252677	0.025385	0.00173
Zn	0.011975	0.072434	0.00095	0.041435

ICP-MS	ttest rock type TP1	ttest rock type TP12	ttest time granite	ttest time gabbro
Al	0.000893	0.084769	0.343015	0.000921
Ti	0.89613	0.767636	0.03253	0.099694
Mn	8.44E-05	0.632249	0.001165	0.157488
Fe	0.004708	0.032869	0.011701	0.008551
Co	0.005175	0.015449	0.001646	0.183184
Ni	0.797786	0.804143	0.002809	0.171669
Cu	1.29E-05	3.12E-05	2.7E-05	0.003091
Zn	0.015057	0.078909	0.001916	0.032414

Figure 3.7 *Significance tests of ICP-OES (left) and ICP-MS (right) data of elemental leaching changes over 18 months, comparing differences between the rock types at time point 1 (first column), differences between the rock types at time point 12 (second column), changes in granite microcosms between time points 1 and 12 (third column), changes in gabbro microcosms between time points 1 and 12 (fourth column). Unpaired t-tests; grey cells significant at 0.05 confidence level.*

The significance tests using unpaired t-tests can be seen in Figure 3.7, where cells marked in grey are those where the changes are significantly different at the 0.05 confidence level. This analysis shows that there are significant differences between the rock types for more elements at the first time point than at the last time point, which indicates that the environments are more similar at the end of the experiment than at the beginning, and thus that the environments are getting more similar over time as the experiment progresses. Furthermore, when comparing the changes within each rock type, granite has significant differences between time points 1 and 12 for more elements than gabbro, indicating that the granite environment changes more than the gabbro microcosms over time.

3.5.1.3 Weather and Seasonal Changes

Changes in temperature and rainfall throughout the course of the experiment are shaping the community assembly process. Weather data was acquired from the weather station at the Royal Botanic Garden Edinburgh, situated 5.4km from the location of the experiment. The daily precipitation can be seen in Figure 3.8 and the daily maximum and minimum temperatures can be found in Figure 3.9.

Clear seasonal variations in temperatures can be observed, although temperature fluctuations are relatively minor as is typical of temperate climates. Daily high temperatures are around 20°C in summer and around 8°C in winter, while daily lows range from about 12°C in summer to just below freezing in winter. The temperature dropped below freezing on 48 days during the course of the experiment, meaning that the communities experienced freezing and thawing events approximately this number of times. Some uncertainty exists as the duration of each freezing event is not known, and samples could freeze and thaw several times a day, or stay frozen for several days in a row.

Seasonal variations in rainfall are not regular, with rainfall throughout the course of the experiment ranging from 0mm per day to 60mm on one day, 6 June 2017. The 6 June 2017 appears to be an anomaly, as days with the next highest amounts of rainfall register about 20mm per day. Of note is a dry spell with only 13.2mm registered between 22 March and 11 May 2017.

3.5.2 Biomass Results

Microcosm biomass was found to increase over time, as evidenced by cell counts under the microscope and CFU counts on 0.2% yeast extract agar plates. Also, it was possible to visually track the increase of biomass through the proxy of macroscopic organisms, such as mosses and algae that appeared after 3 months and continued to increase in mass over time.

3.5.2.1 Cell counts

Cell numbers generally increase until about Month 11, at which point the numbers plateau around 2×10^5cells/gram rock, while at the final month both rock types have roughly 2.5×10^5cells/gram rock (Figure 3.10). Some fluctuation is observed

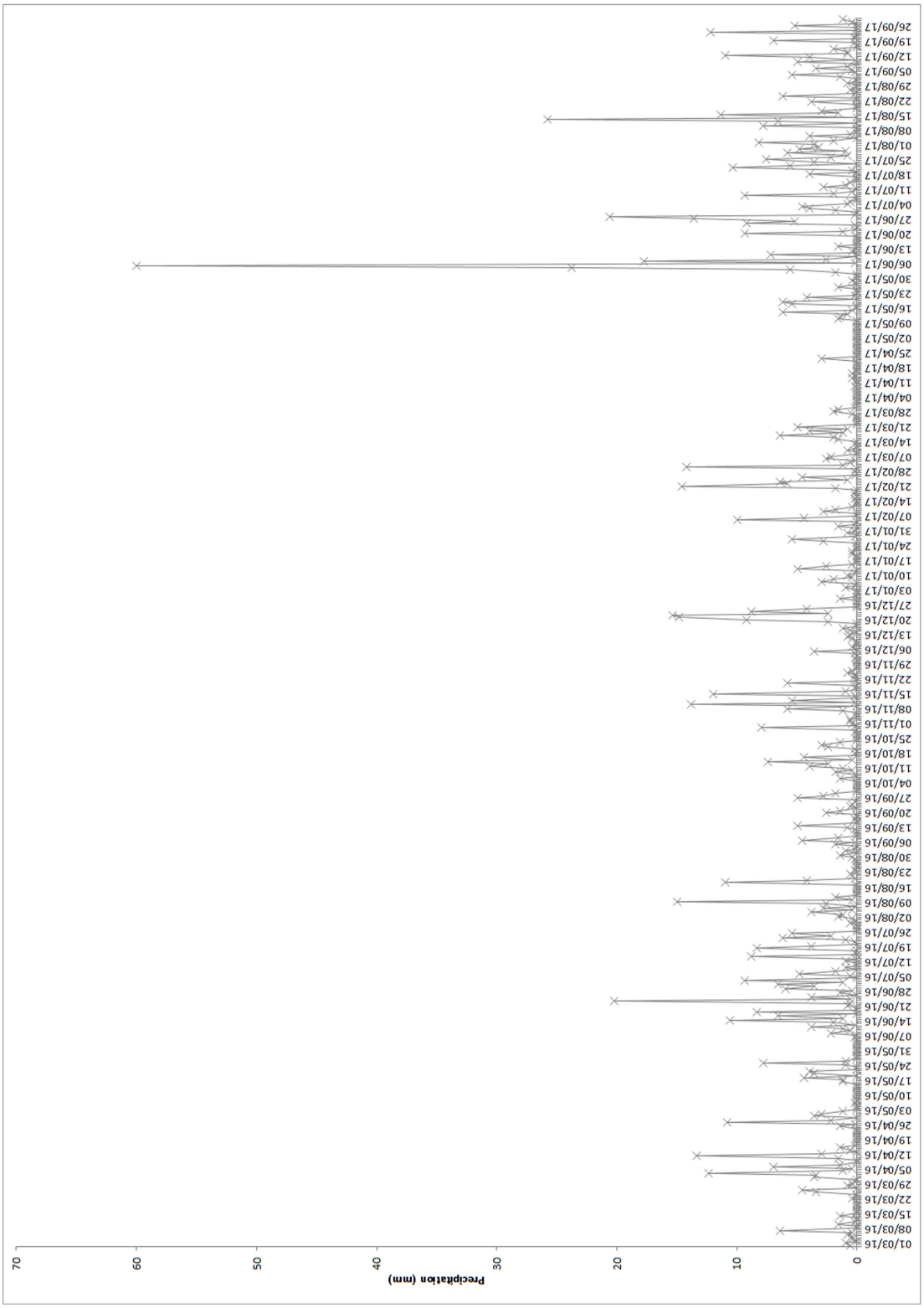


Figure 3.8 *Daily rainfall during the course of the experiment, as measured in mm by the weather station at the Royal Botanic Garden Edinburgh.*

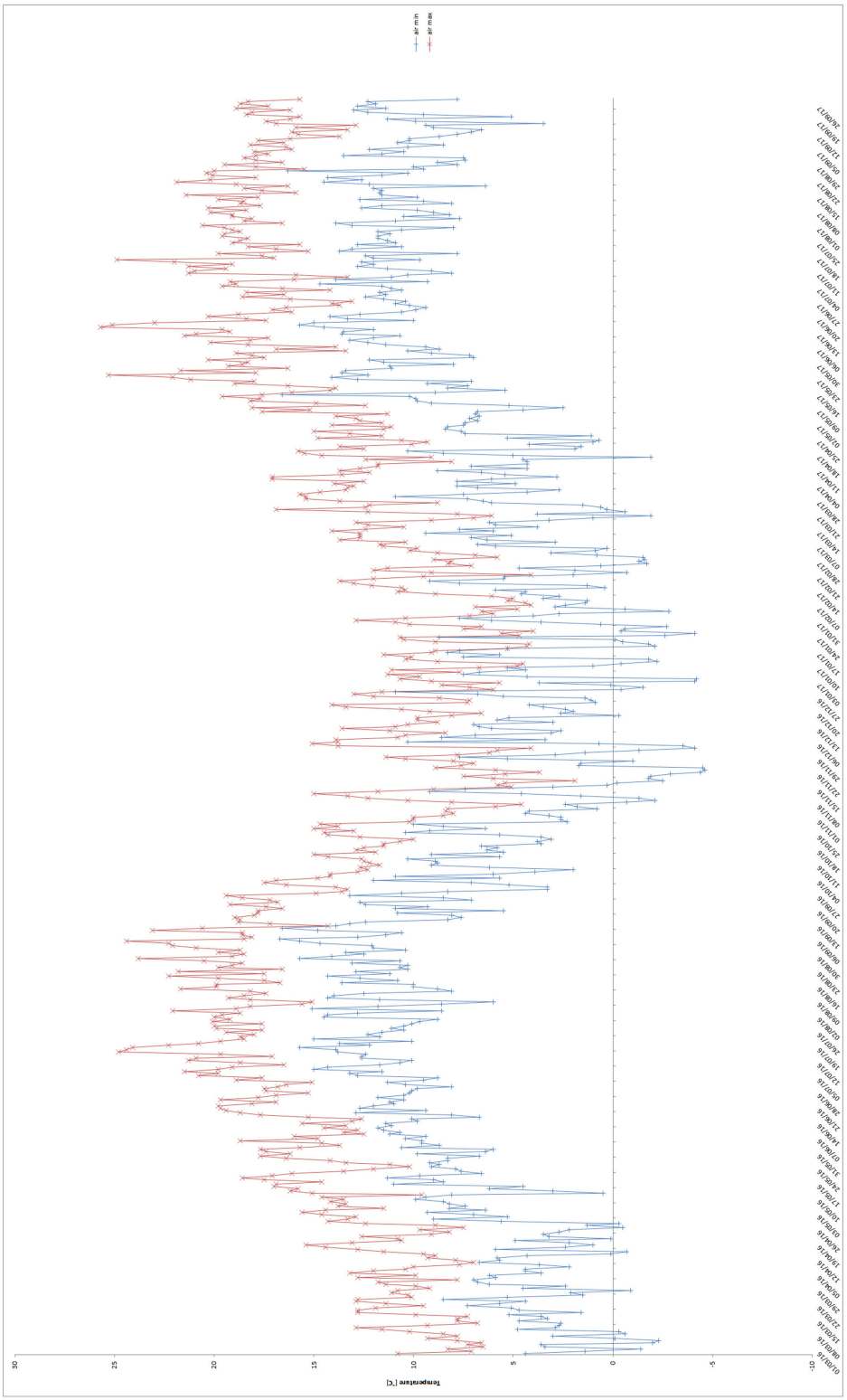


Figure 3.9 *Daily maximum and minimum temperatures during the course of the experiment, as measured in degrees Celsius by the weather station at the Royal Botanic Garden Edinburgh. Red=daily max, blue=daily min.*

such that the cell numbers are not strictly increasing, which may be due to seasonal variations such as differences in rainfall and temperature. The increases seen in cell numbers over time are statistically significant at the 0.05 level for both granite (t-test, $p=2.39 \cdot 10^{-23}$) and gabbro (t-test, $p=2.98 \cdot 10^{-23}$). The difference in cell counts between rock types is statistically significant at the 0.05 confidence level at Month 1 (t-test, p=0.00928) but not at Month 18. Biomass influx from immigration into the system can be seen in Figure 3.11, tracking cell counts for the intermediate control samples (Section 3.3.12), showing that there is some variation in the amount of immigration biomass each month, but that overall the variation is small. Note also that for the later time points the sampling interval gets longer (exposure time for the intermediate samples are one month up until Month 7, two months up until Month 15, and 3.5 months for the final sampling interval). Micrographs from Month 18 are shown in Figure 3.12.

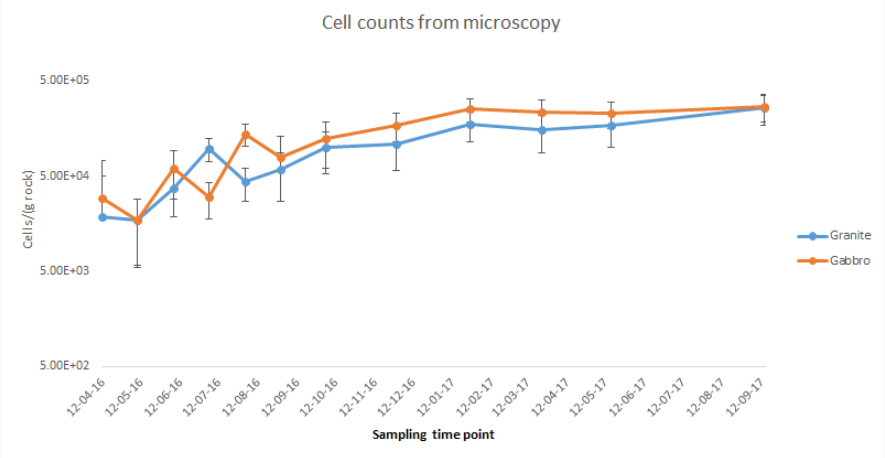


Figure 3.10 *Cell counts by microscopy over 18 months. Error bars standard error (n=3).*

3.5.2.2 CFU counts and observations

CFU (colony forming unit) counts were conducted on 0.2% yeast extract agar plates. For each time point, CFU counts, morphology types and colours were monitored. Data was collected after 1, 5 and 7 days of incubation, in order to monitor the number of opportunitists, i.e. fast growers, compared to the total number of colonies. The counts after 7 days are shown in Figure 3.13. Throughout the course of the experiment the number of cells per gram rock increases from about 10^6 to 10^{10}, in both granite and gabbro, with the concentrations being closely correlated between the rock types, indicating that they harbour a similar-sized community. These results were found to be just outside statistically

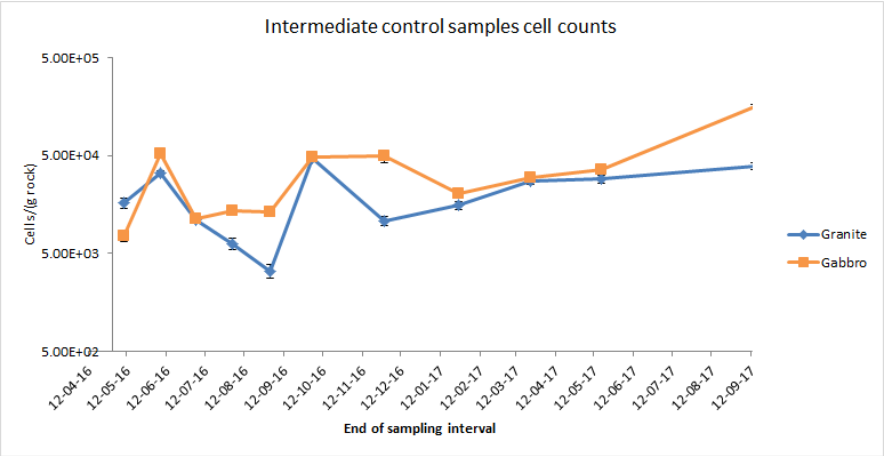
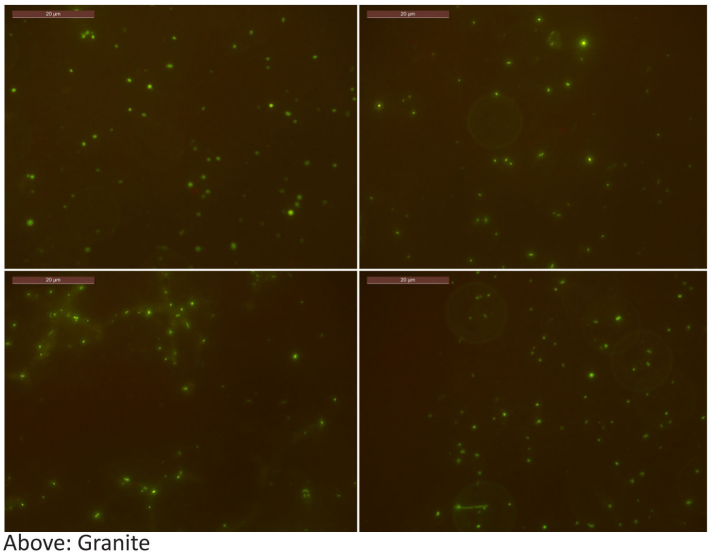


Figure 3.11 *Cell counts by microscopy for intermediate control samples over 18 months. Each data point represents the cell counts of the intermediate control samples that were inoculated between that time point and the preceding time point. The sampling intervals or exposure time for intermediate samples are one month up until Month 7, two months up until Month 15 and 3.5 months for the final sampling interval. Error bars standard error (n=600).*

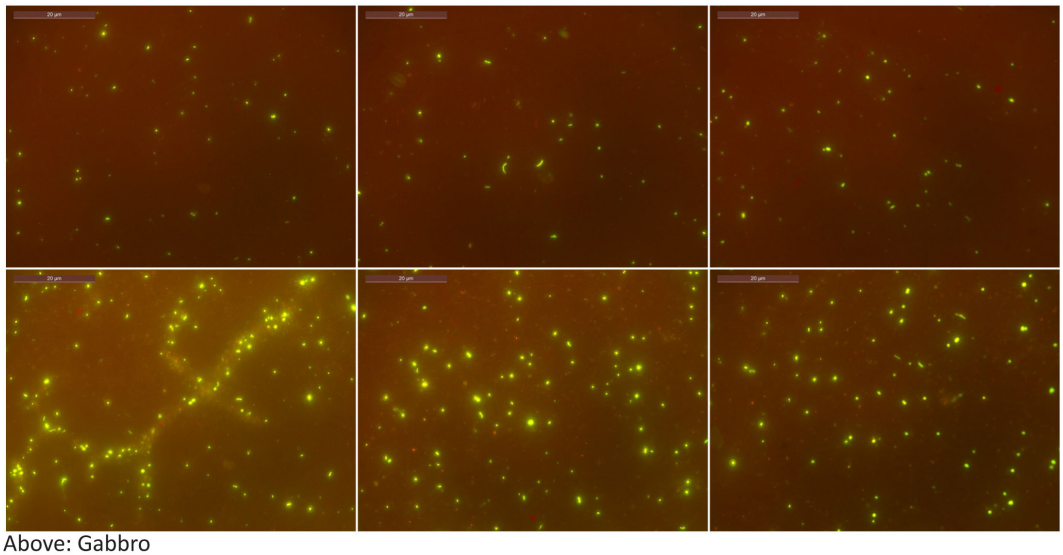
significant at the 0.05 confidence level for the changes between Months 1 and 18 for both granite (t-test, p=0.0662) and gabbro (t-test, p=0.0158), although the averages have visibly increased over time.

The CFU counts must be taken with some caution, as dilution series were carried out in order to quantify the number of CFUs. It was noted that often a ten-fold dilution did not result in a ten-fold decrease in CFUs, likely attributed to the fact that the community is not homogeneous and that even with standard mixing procedures dilutions are not linear. It is also possible that various types of organisms grow better at different concentrations as they are spread on the plates, and that some organisms are not successful at growing if the plates are overcrowded, but can grow successfully at higher dilutions.

No visible increase in number of colony types was seen on the agar plates, contrary to the results of the 16S rRNA sequencing which indicate that sample diversity is increasing throughout the experiment, however, it is possible that the culturable fraction remained relatively constant. It is also possible that the number of distinguishable colonies did not increase, while the de facto diversity increased. Causes for this mismatch between diversity observations from solid culturing and molecular analysis could be the fact that only a small part of the community is culturable on solid media, and it is possible that this fraction did not increase significantly over the course of the experiment. Another explanation might be



Above: Granite



Above: Gabbro

Figure 3.12 *Micrograph of cell counts at the final time point for granite (top) and gabbro (bottom).*

that the diversity which can be observed on an agar plate was reached early on, as each plate only has a finite surface area where colonies can grow, and thus a relatively limited diversity can be displayed in what may otherwise be a highly diverse sample. With the tests conducted, it was not possible to answer this question further.

There was also no significant increase in the number of colours observed among the colonies. The main colours observed were white/cream, clear/transparent, yellow, orange, red, light pink, hot pink and purple. Purple colonies were observed to a greater extent during certain months (time points 1, 2, 3, 4, 6; to a lesser extent time points 5, 8, 9), while most other colours were observed every month. Hot

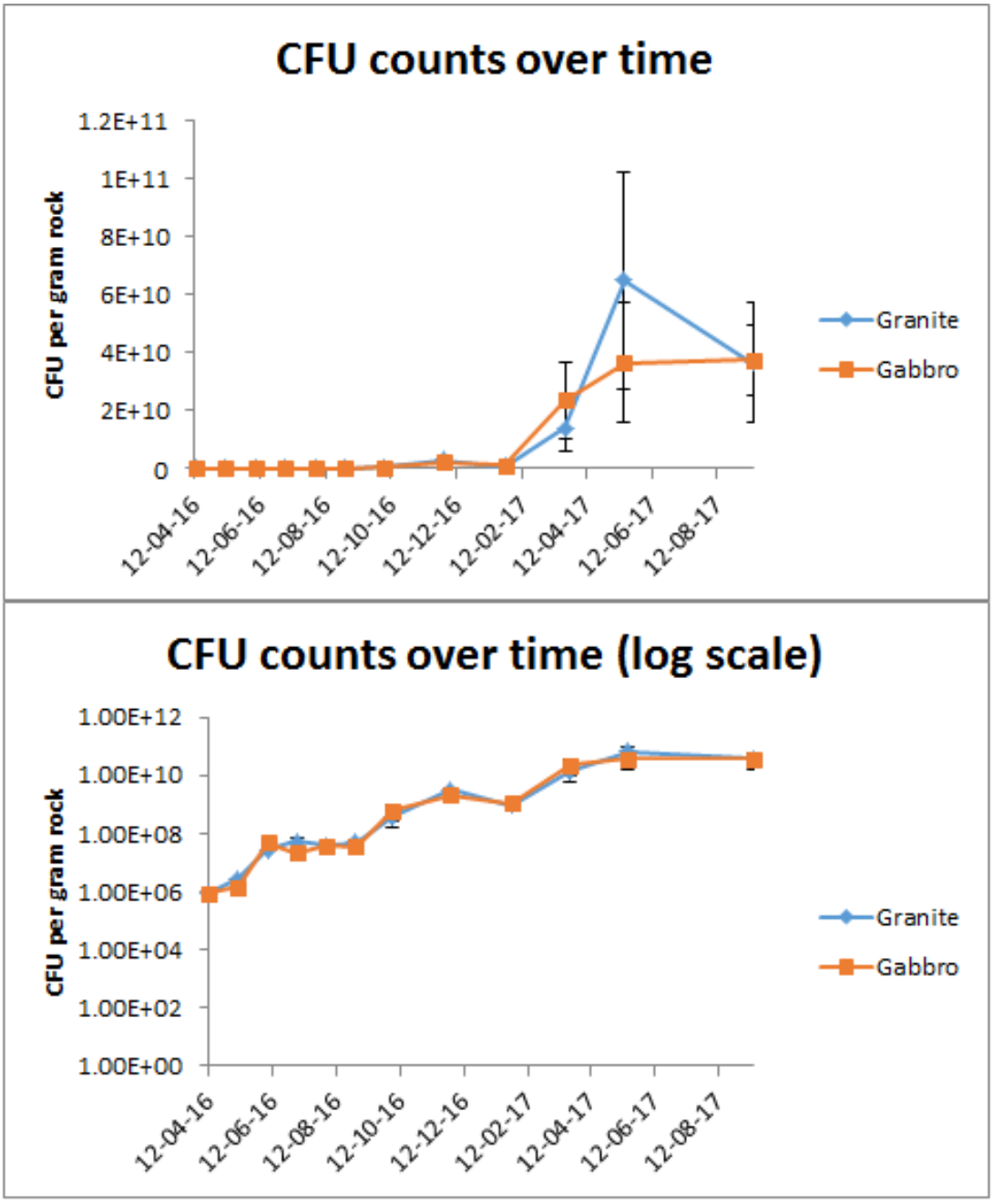


Figure 3.13 *CFU counts on yeast extract agar as a function of time, showing the same results for both linear scale (top) and logarithmic scale (bottom). Error bars are standard error (n=9).*

pink colonies generally only occurred after 7 days of incubation on agar plates, while most other colours were visible after 5 days.

An attempt was made to culture and identify the purple colonies that were ubiquitous in the first few months of the experiment, but this was unsuccessful - the purple colonies appeared to be growing exclusively inside other white colonies, and any attempts to transfer and separate these strains resulted in only white colonies being transferred. The purple colonies were found to be very hard, and thus it was difficult to scrape off any cells in order to complete the transfer.

3.5.2.3 DNA concentration from qPCR

DNA concentration from qPCR (copies/gram rock) are shown in Figures 3.14 and 3.15 (log scale), and act as a proxy for the amount of biomass in each sample. This interpretation must be treated with some caution however, as it is not known how many DNA copies each cell contains, and the efficiency of extraction and amplification is unknown. There is a significant increase (at 0.05 confidence level) between the DNA concentration at Months 1 and 18 for both granite (t-test, p=0.00456) and gabbro (t-test, p=0.0160). At both the beginning (Month 1, t-test, p=0.000818)) and the end (Month 18, t-test, p=0.000258) of the experiment, there are significant differences in the DNA concentration between the two rock types, with a higher DNA concentration in gabbro at Month 1, while granite has s higher DNA concentration at Month 18.

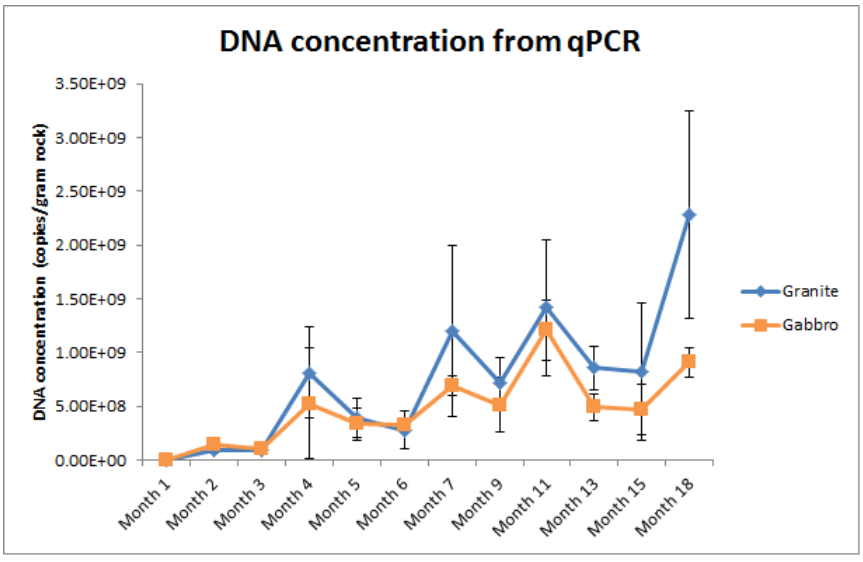


Figure 3.14 *DNA concentration as a proxy for biomass as measured over time. Error bars are standard error (n=9).*

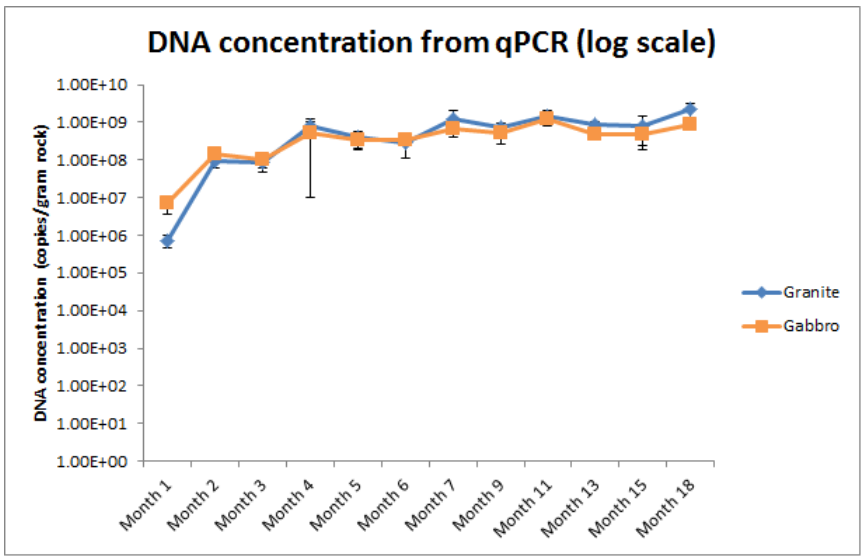


Figure 3.15 *DNA concentration as a proxy for biomass as measured over time (logarithmic scale). Error bars are standard error (n=9).*

3.5.3 Phototroph culturing results

Phototrophs are an important part of many rock-dwelling communities where sunlight is present, as they provide an *in situ* carbon source through photosynthesis, which is made available to the rest of the community to utilise. In this study, phototroph emergence was monitored throughout the experiment, in order to determine at which point they may become an important part of the community ecosystem. Thus, phototroph culturing was conducted at each sampling time point, to study whether phototrophs were in principle present in the sample at that time (see Section 3.3.13).

Algae and cyanobacteria were observed in all samples at all time points after phototroph culturing. This shows that phototrophs are present in the samples right from the start during the first month. They do not initially show up in significant numbers in the DNA sequencing results, as they are are slow-growing and hence not present in large enough quantities to show up in the sequencing until Month 3. Some differences are seen between the culturable species during the time course of the experiment - Month 1 has mostly small, coccoidal cells (Figures 3.16 and 3.17), whereas Month 18 is dominated by long, filamentous cyanobacteria and algae (Figures 3.18 and 3.19). The sole aim of this test was to establish when phototrophs appeared in the samples, and no further tests were conducted on the phototrophs once it was established that they were present in the samples from all time points.

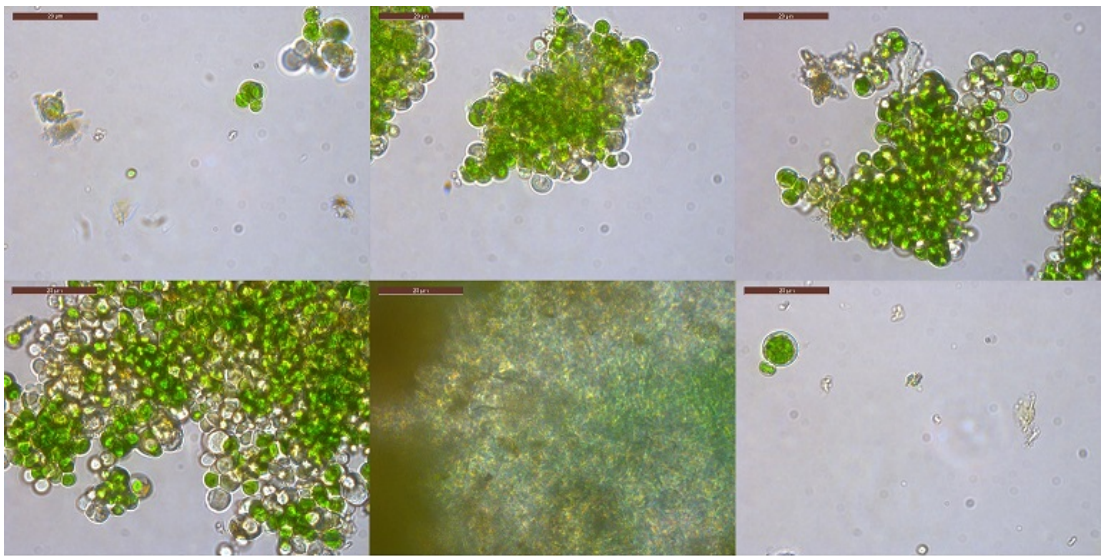


Figure 3.16 *Micrographs of phototrops from granite samples at Month 1. Scale bars are* $20\mu m$ *long.*

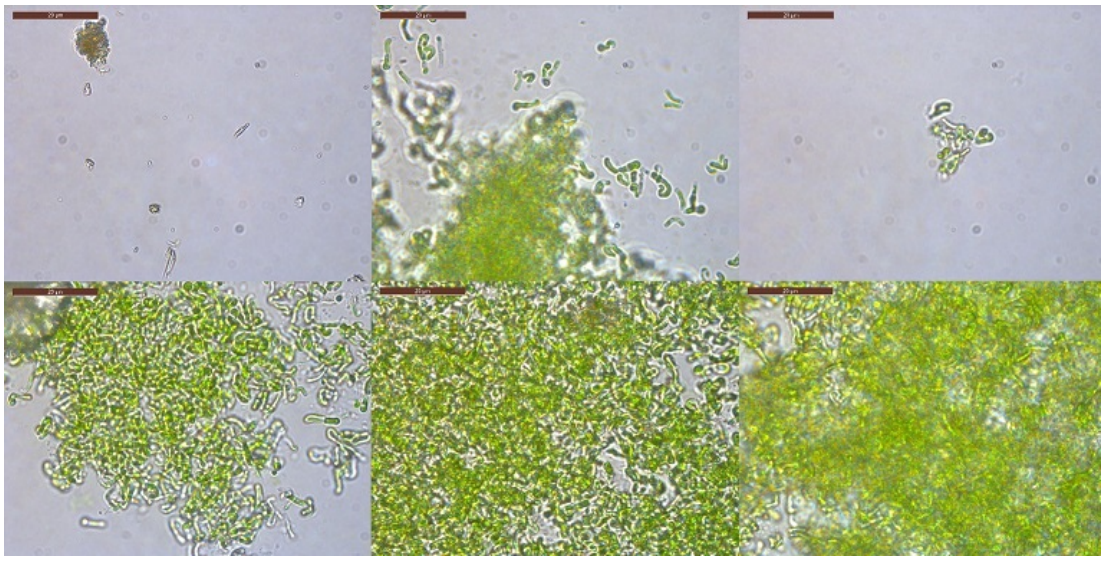


Figure 3.17 *Micrographs of phototrops from gabbro samples at Month 1. Scale bars are* $20\mu m$ *long.*

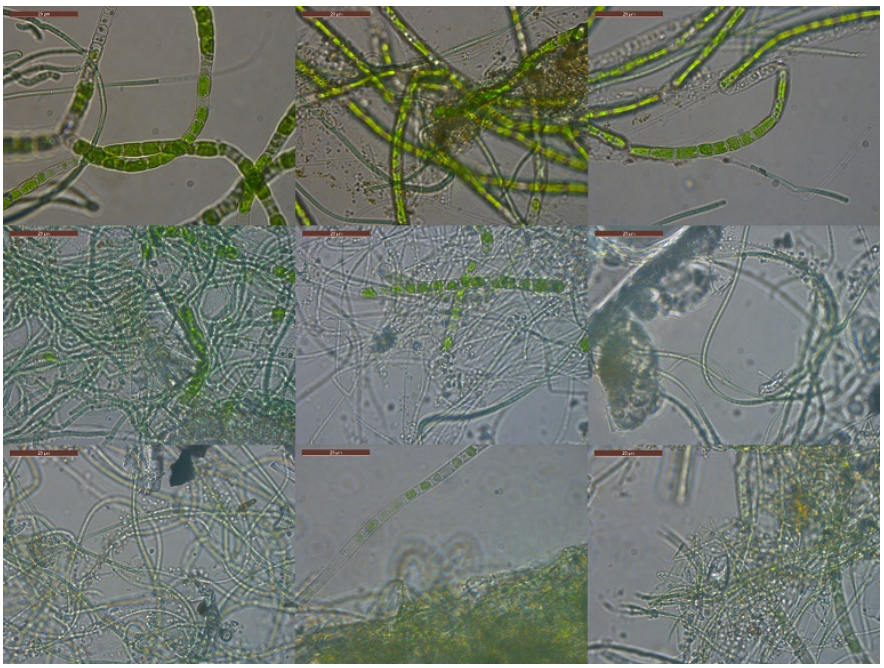


Figure 3.18 *Micrographs of phototrops from granite samples at Month 18. Scale bars are* $20\mu m$ *long.*

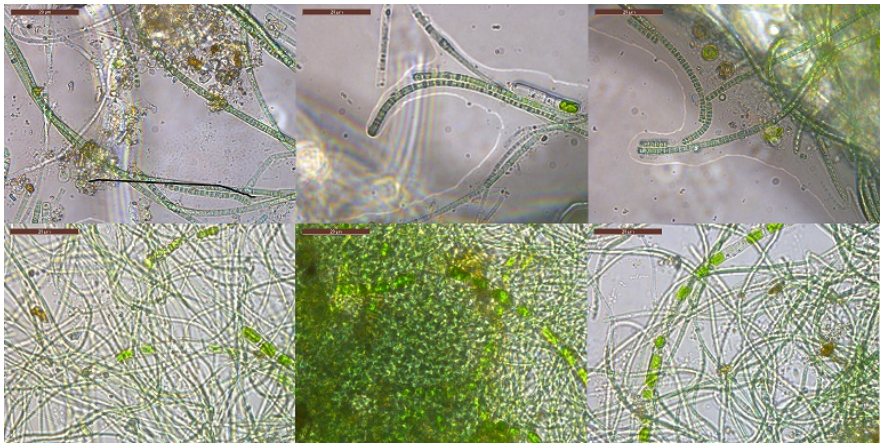


Figure 3.19 *Micrographs of phototrops from gabbro samples at Month 18. Scale bars are* $20\mu m$ *long.*

3.5.4 Community Composition Results

The composition of the microbial communities was revealed by an analysis of the 16S rRNA data using Qiime (Section 3.4). The communities are similar but distinct between the rock types, and increase in similarity and diversity over time.

3.5.4.1 Community Composition - Stacked Bar Charts

Community data is shown here at Phylum, Class and Genus level. Phylum level gives the least granular overview of the community, and reveals what the major components of the community are. At Class level, these phyla are broken down further, and it is possible to observe the relative abundance of the different types of Proteobacteria, predominantly Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria. Genus level then gives the most comprehensive overview of the community, at the most detailed level identifiable from the data. Graphs for Order and Class were not included, as these were not deemed to add significant detail to answer the questions being posed about the community.

3.5.4.1.1 Phylum Level Phylum level composition is shown in Figure 3.20. The early community is dominated by Proteobacteria, while later on Cyanobacteria becomes the most abundant taxon. The small fraction of Bacteriodetes increases somewhat but stays small, below 25%, and a small percentage of Gemmatimonadetes appears at Month 3 in gabbro and Month 4-5 in granite. Overall, the communities appear to be very similar in granite and gabbro.

These data are somewhat easier to interpret by using line graphs depicting the changes in relative abundance of each phylum. The increases in Cyanobacteria and Bacteriodetes relative abundance can be seen in Figures 3.21 and 3.22, respectively. The changes in relative abundance of Cyanobacteria between Months 1 and 18 are statistically significant at the 0.05 confidence level for both granite (t-test, p=0.00248) and gabbro (t-test, p=0.00366), as is the case for Bacteroidetes for granite (t-test, p=0.0340) and gabbro (t-test, p=0.0351).

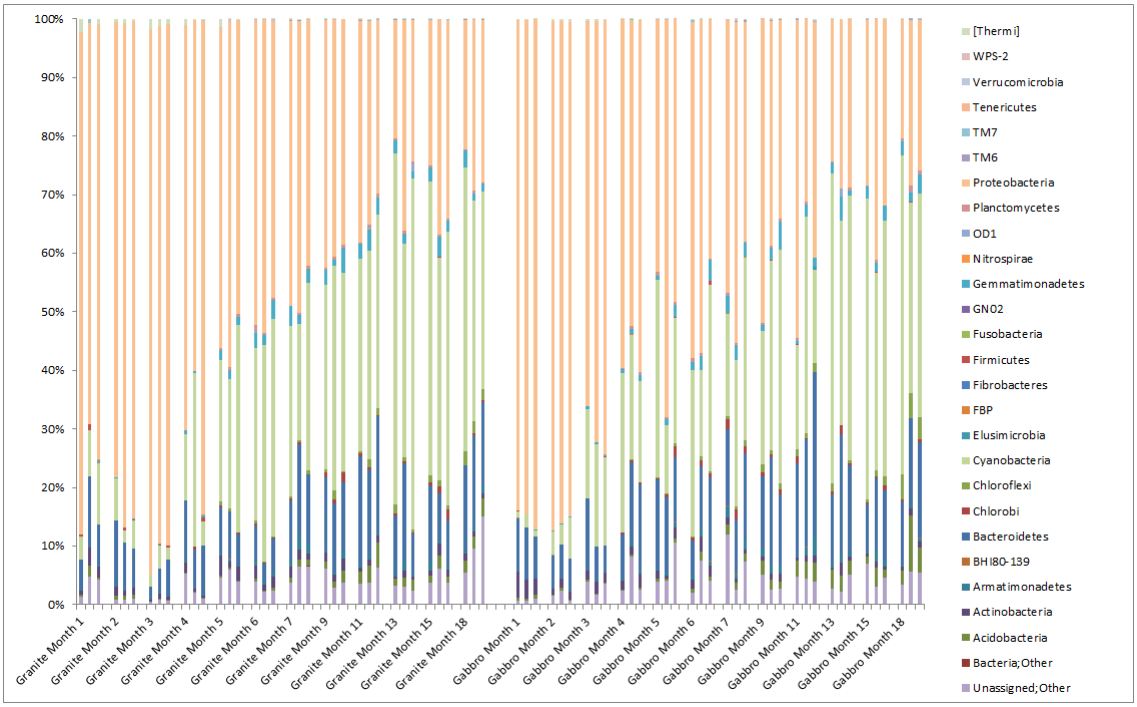


Figure 3.20 *Phylum level microbial community composition in both rock types over 18 months. Samples are ordered in two blocks, with granite on the left and gabbro on the right. For each rock type, triplicate samples are organised left to right according to time point, with the earliest time point on the left. The three most dominant phyla are Proteobacteria in orange, Cyanobacteria in green, and Bacteroidetes in blue.*

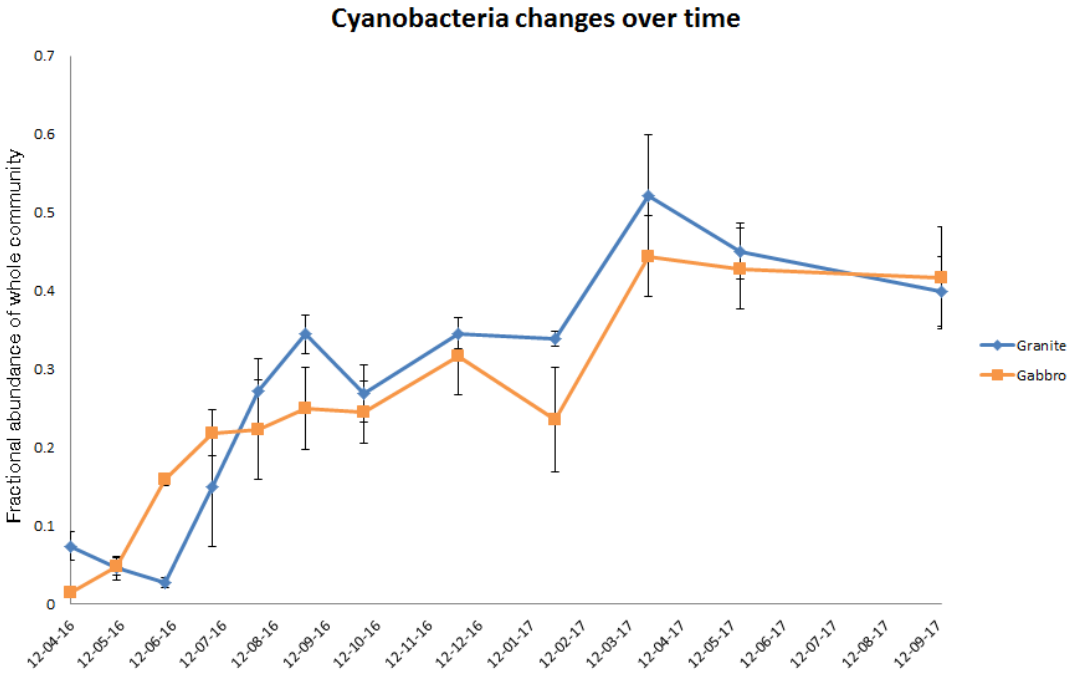


Figure 3.21 *Changes in Cyanobacteria relative abundance in both rock types over 18 months. Error bars are standard error (n=3).*

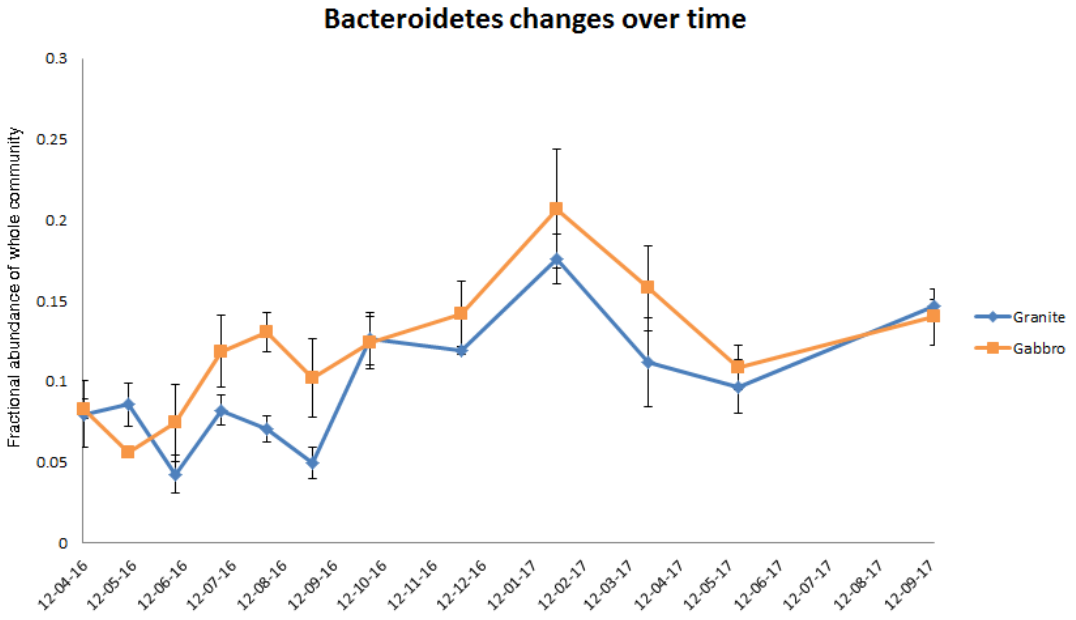


Figure 3.22 *Changes in Bacteroidetes relative abundance in both rock types over 18 months. Error bars are standard error (n=3).*

3.5.4.1.2 Class Level At class level, some more patterns in the community composition become visible (Figure 3.23), showing the contribution to Proteobacteria by its various classes and how these change over time. It also distinguishes between chloroplast 16S DNA in eukaryotes (see Section 3.5.5) and the Cyanobacteria class Synechococcophycideae. The most dominant taxa seen at class level are, from the top to the bottom of the graph, Gammaproteobacteria (light blue), Betaproteobacteria (yellow), Alphaprotebacteria (pinkish-red), Cyanobacteria class Synechococcophycideae (military green), chloroplast 16S DNA (bright green), Bacteroidetes class Saprospirae (dark red), Bacteroidetes class Cytophagia (dark blue). Figure 3.24 displays the Proteobacteria classes' relative abundance over time. Decreases are observed in both Alphaproteobacteria and Betaproteobacteria over time that are statistically significant at the 0.05 confidence level (Alphaproteobacteria granite: t-test, p=0.000618; Betaproteobacteria granite: t-test, p=0.0110; Alphaproteobacteria gabbro: t-test, p=0.00495; Betaproteobacteria gabbro: t-test, p=0.000489).

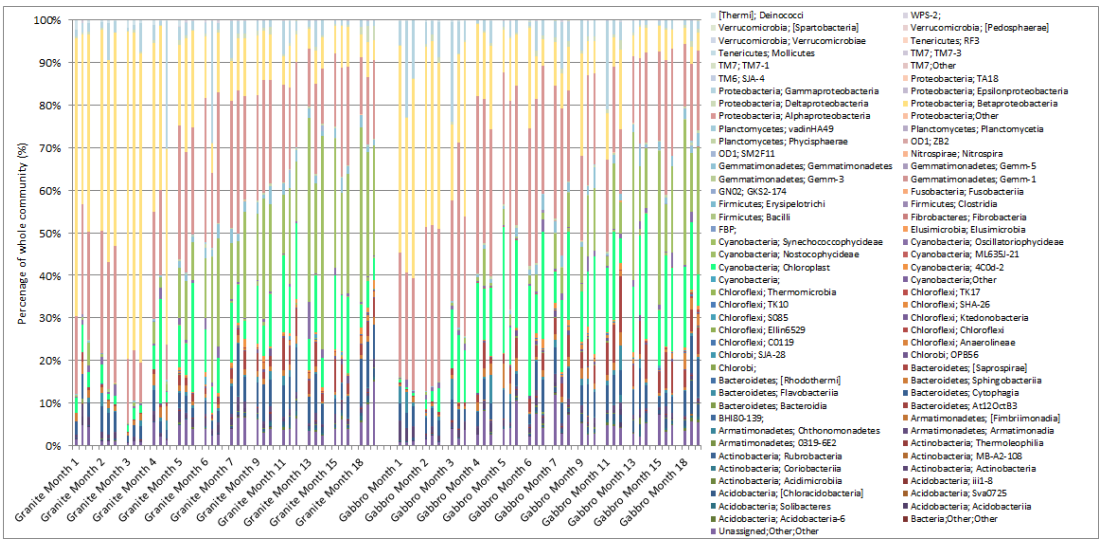


Figure 3.23 *Class level microbial community composition in both rock types over 18 months. Samples are ordered in two blocks, with granite on the left and gabbro on the right. For each rock type, triplicate samples are organised left to right according to time point, with the earliest time point on the left. The most dominant taxa seen at class level are, from the top to the bottom of the graph, Gammaproteobacteria (light blue), Betaproteobacteria (yellow), Alphaprotebacteria (pinkish-red), Cyanobacteria class Synechococcophycideae (military green), chloroplast 16S DNA (bright green), Bacteroidetes class Saprospirae (dark red), Bacteroidetes class Cytophagia (dark blue).*

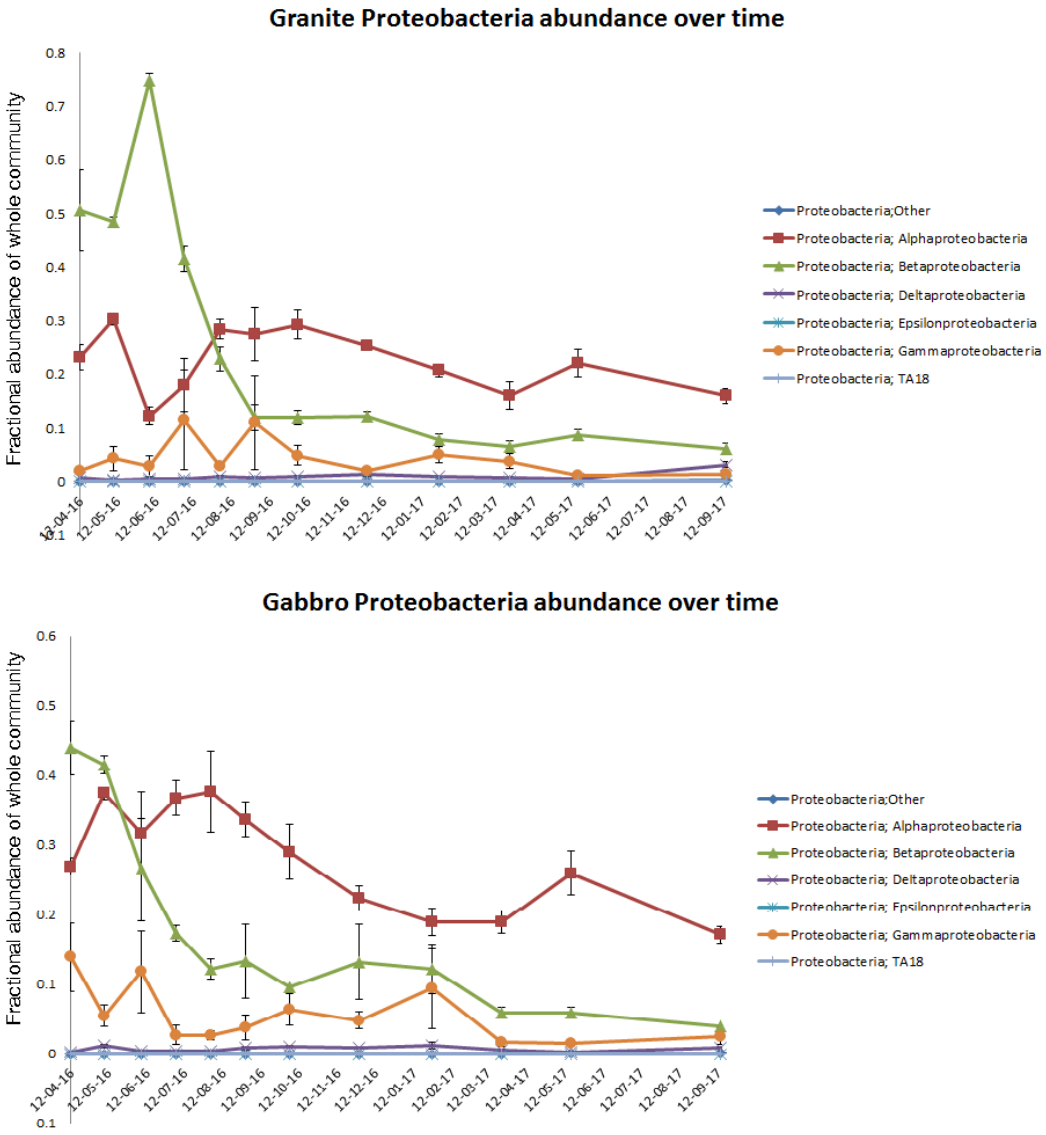


Figure 3.24 *Changes in Proteobacteria classes relative abundance in both rock types over 18 months. Error bars are standard error (n=3).*

3.5.4.1.3 Genus Level At genus level, the number of OTUs and hence diversity in each sample are observed (Figure 3.25), showing that the diversity in the communities increases over time. The genera with highest relative abundance early on are *Janthinobacterium* (light blue), Oxalobacteraceae family (yellow), *Sphingomonas* (dark blue) and *Rhizobiaceae* (purple), whereas later on the largest relative abundance is *Leptolyngbya* (dark purple) and *Stramenopiles* algae (light green).

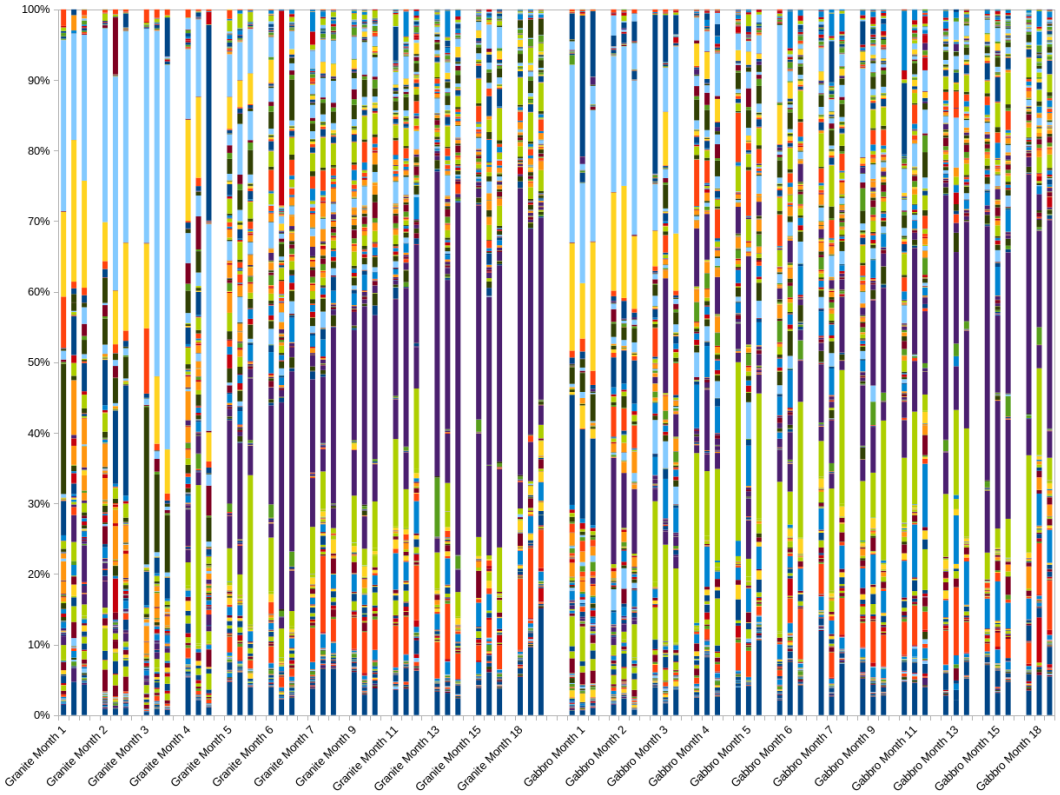


Figure 3.25 *Genus level microbial community composition in both rock types over 18 months. Samples are shown in triplicate per time point and rock type, each column representing one sample. Samples are ordered in two blocks, with granite on the left and gabbro on the right. For each rock type, triplicate samples are organised left to right according to time point, with the earliest time point on the left. The genera with highest relative abundance early on are Janthinobacterium (light blue), Oxalobacteraceae family (yellow), Sphingomonas (dark blue) and Rhizobiaceae (purple), whereas later on the largest relative abundance is taken up by Leptolyngbya (dark purple) and Stramenopiles algae (light green).*

Figure 3.26 shows the abundance of *Janthinobacteria* during the course of the experiment, both as a relative abundance of the community (top) and as an absolute value as calculated using the qPCR data (bottom). The relative abundances are a relatively good proxy for the granite absolute abundances, and to a lesser extent for gabbro, apart from the first few months for gabbro, where the relative abundance is strictly decreasing while the absolute abundance fluctuates. Changes in relative abundance over time are statistically significant at the 0.05 confidence level for both granite (t-test, p=0.00175) and gabbro (t-test, p=0.00389), while changes in absolute abundance are not. Differences between the rock types are statistically significant at the 0.05 confidence level for absolute abundance at Month 1 (t-test, p=0.00572).

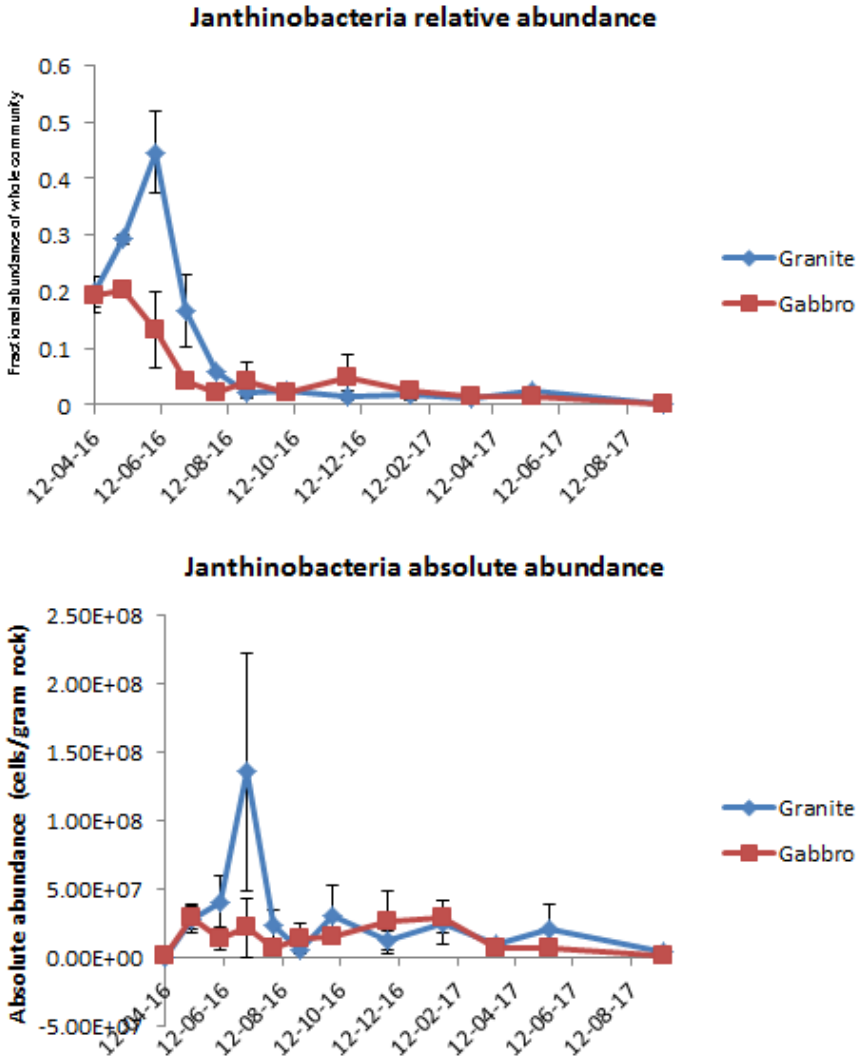


Figure 3.26 *Janthinobacteria relative abundance (top) and absolute abundance (bottom) as a function of time during the experiment.*

Figure 3.27 shows the abundance of *Sphingomonas* during the course of the experiment, both as a relative abundance of the community (top) and as an absolute value as calculated using the qPCR data (bottom). Here, the profiles are remarkably different between relative and absolute abundance, as the relative abundance decreases after the early months and then stays relatively constant, whereas the absolute abundance shows that *Sphingomonas* is in fact fluctuatingly increasing during the experiment. Changes in relative abundance over time are statistically significant at the 0.05 confidence level for both granite (t-test, p=0.000981) and gabbro (t-test, p=0.000366), as are changes in absolute abundance for granite (t-test, p=0.000574) and gabbro (t-test, p=0.0134). Differences between the rock types are statistically significant at the 0.05 confidence level for both relative (t-test, p=0.00157) and absolute abundance (t-test, p=0.000246) at Month 1.

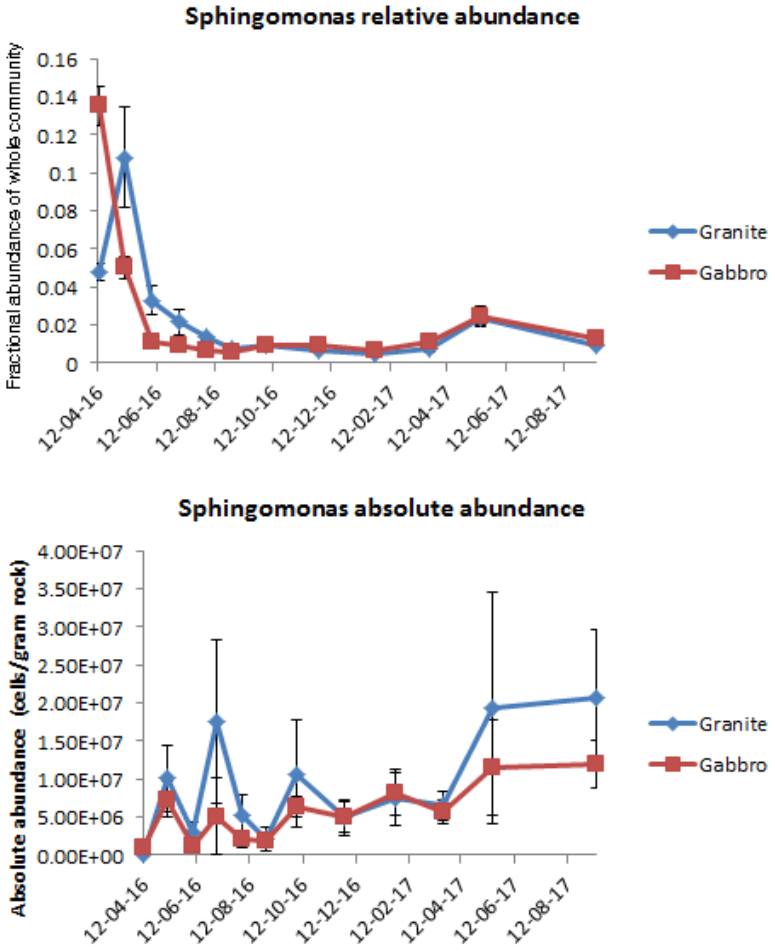


Figure 3.27 *Sphingomonas relative abundance (top) and absolute abundance (bottom) as a function of time during the experiment.*

Figure 3.28 shows the abundance of *Leptolyngbya* during the course of the experiment, both as a relative abundance of the community (top) and as an absolute value as calculated using the qPCR data (bottom). Here, the profiles are relatively similar between relative and absolute abundance, as both show a large increase during the course of the experiment, but there is a larger increase in granite absolute abundance between the final two time points than what the relative abundance reveals. Changes in relative abundance over time are statistically significant at the 0.05 confidence level for both granite (t-test, p=0.00336) and gabbro (t-test, p=0.01393), as are changes in absolute abundance for granite (t-test, p=0.00211) and gabbro (t-test, p=0.0139). Differences between the rock types are statistically significant at the 0.05 confidence level for the absolute abundance at Month 18 (t-test, p=0.0135).

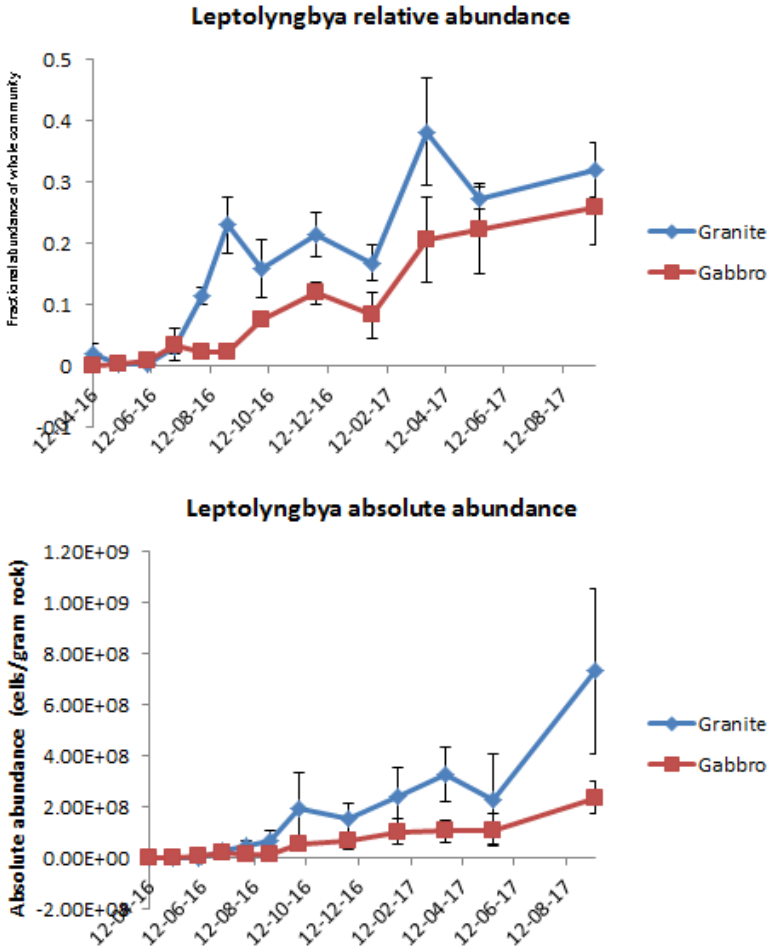


Figure 3.28 *Leptolyngbya relative abundance (top) and absolute abundance (bottom) as a function of time during the experiment.*

3.5.4.1.4 Most abundant OTUs In order to better understand the make-up of the community at genus level, the 40 most common OTUs from across all samples are shown (Figure 3.29). These were calculated first without the intermediate control samples, as including these would bias the selection toward the early community, however, when calculating the 40 most common taxa while including the intermediate control samples, only 5 taxa were different, so these additional 5 taxa from the intermediate samples were also included in the list.

Without intermediate control samples
Cyanobacteria;c__Synechococcophycideae;o__Pseudanabaenales;f__Pseudanabaenaceae;g__Leptolyngbya
Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__Janthinobacterium
Cyanobacteria;c__Chloroplast;o__Stramenopiles;f__;g__
Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__
Cyanobacteria;c__Chloroplast;o__Streptophyta;f__;g__
Unassigned;Other;Other;Other;Other;Other
Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae;g__
Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__
Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingomonas
Bacteroidetes;c__[Saprospirae];o__[Saprospirales];f__Chitinophagaceae;g__
Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__
Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas
Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;Other
Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__;g__
Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__
Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Rhodobacter
Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Pelomonas
Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Acetobacteraceae;g__
Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__Methylobacterium
Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;g__Mycoplana
Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__Flavobacterium
Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae;g__
Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;Other
Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;g__Phenylobacterium
Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Devosia
Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Paucibacter
Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__;g__
Gemmatimonadetes;c__Gemmatimonadetes;o__Gemmatimonadales;f__Gemmatimonadaceae;g__Gemmatimonas
Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__
Cyanobacteria;c__Oscillatoriophycideae;o__Oscillatoriales;f__Phormidiaceae;g__Phormidium
Armatimonadetes;c__Armatimonadia;o__Armatimonadales;f__Armatimonadaceae;g__
Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Dokdonella
Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__Agrobacterium
Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae;g__Bosea
Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__;g__
Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae;g__Spirosoma
Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;Other;Other
Acidobacteria;c__[Chloracidobacteria];o__RB41;f__Ellin6075;g__
Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;Other
Gemmatimonadetes;c__Gemmatimonadetes;o__Gemmatimonadales;f__A1-B1;g__
With intermediate control samples, 5 most common taxa not included above
Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Variovorax
Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__Sphingobacteriaceae;g__Pedobacter
Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Sinobacteraceae;g__Nevskia
Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae;g__Dyadobacter
Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__Sphingobacteriaceae;g__

Figure 3.29 *A list of the 40 most abundant taxa across all samples.*

Of the 40 most abundant taxa, only 18 (less than half) are defined down to genus level, while other taxa are sequences that have been grouped at a higher level. Of the top 10, only 3 genera are defined. This indicates that potentially a large number of sequences found in the samples are from organisms that are

not well characterised. The most abundant defined genera, when totalling up the presence in all samples, are *Leptolyngbya*, *Janthinobacterium*, *Sphingomonas*, *Pseudomonas*, *Rhodobacter*, *Pelomonas*, *Methylobacterium*, *Mycoplana*, *Flavobacterium* and *Phenylobacterium*, of which 8 are Proteobacteria, 1 is Cyanobacteria and 1 is Bacteriodetes.

3.5.4.1.5 Intermediate Control Samples The community composition of the intermediate control samples show that the immigration into the system is relatively unchanged throughout the year, such that the changes in the communities of the main microcosms cannot be explained by seasonal variation in immigration. Figure 3.30 displays both the phylum and genus level breakdown of these monthly control samples. Note that the final sampling interval was longer, meaning that the final intermediate samples were given 3.5 months for colonisation, and accordingly look more diverse than the other samples.

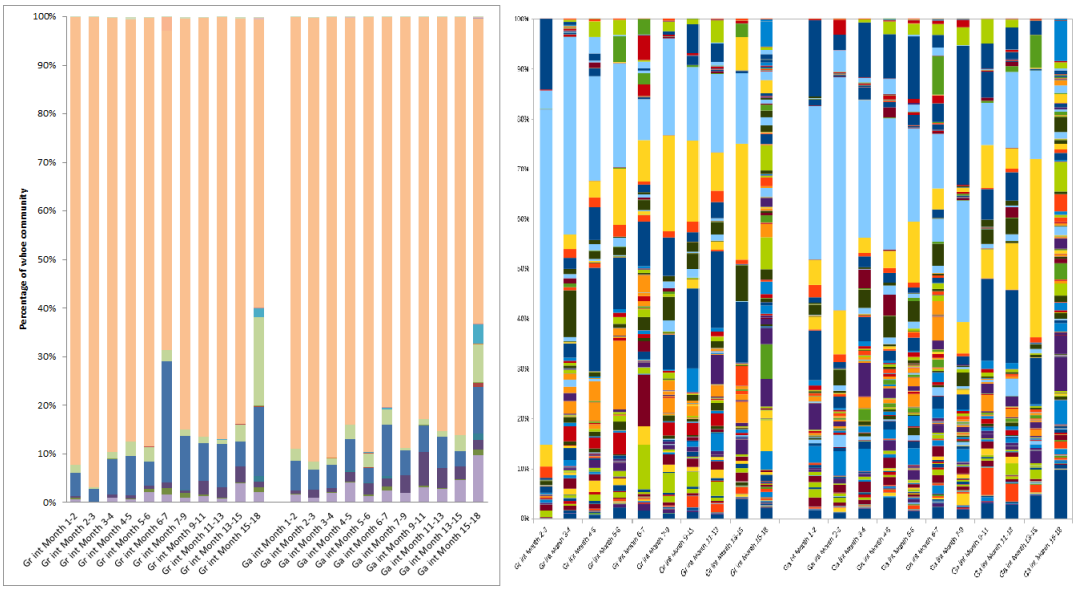


Figure 3.30 *Monthly intermediate control samples community composition in both rock types for phylum (left) and genus level (right). Within these two sections, samples are ordered in two blocks, with granite on the left and gabbro on the right. For each rock type, the samples are organised left to right according to the time point, with the earliest time point on the left.*

3.5.4.2 Alpha Diversity

Alpha diversity was quantified using several different metrics, which all reveal something slightly different about the sample set (Figure 3.31). Chao1 and

Observed OTUs follow the same pattern and show a small but fluctuating increase in diversity, while the Simpson and Shannon indices indicate no net increase in diversity, but with some dips in diversity at Months 3 and 13. Although a cut-off for the rarefaction depth of 9300 was used, the true range of sequences in the samples stretched from 9300 to 200000, with between 10000-80000 sequences identified in the majority of samples. Figure 3.32 shows that on average the number of observed otus are higher for gabbro than granite.

Figure 3.31 *Alpha diversity metrics: Chao1, Observed OTUs, Simpson index and Shannon index over time for both rock types.*

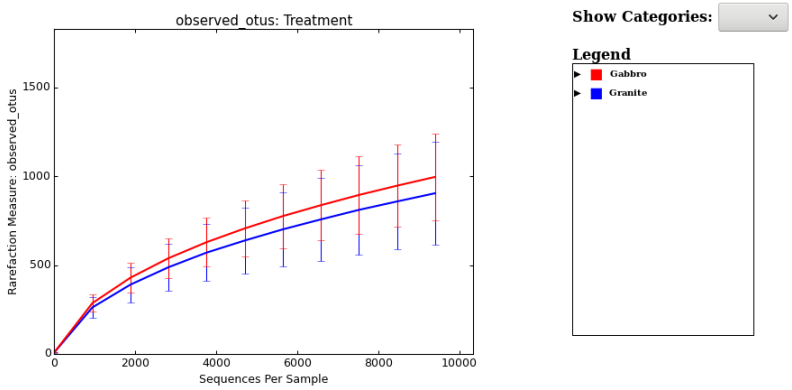


Figure 3.32 *Alpha diversity metric Observed OTUs averaged over all samples per rock type.*

3.5.4.3 Beta Diversity

Beta diversity was analysed using Qiime by producing PCoA plots using Weighted and Unweighted UniFrac, enabling analysis of clustering according to both rock type and time (see Figures 3.33, 3.34, 3.37 and 3.38).

3.5.4.3.1 Rock Type Figures 3.33 and 3.34 show how the microbial communities cluster according to rock type, where granite is shown in blue and gabbro in red, for Unweighted and Weighted UniFrac respectively. From the plot of Unweighted UniFrac, which takes into account only presence and absence of taxa, it appears that the samples are clustering according to rock type right from the start of the experiment (left side of graph) and that they stay separate until the final few time pojnts, although the communities start overlapping somewhat already around Month 6-7. For the Weighted UniFrac analysis, which takes into account OTU abundance, the samples overlap more according to rock type, but the samples instead cluster in the third dimension along the third principal component, although the separation is not as strong as for Unweighted UniFrac. The principal coordinates have values 19.85%, 5.38% and 3.66% for Unweighted UniFrac and 47.58%, 8.13% and 5.89% for Weighted UniFrac. The third dimension of the graphs is illustrated in Figures 3.35 and 3.36, where the graphs are viewed from the PC1 axis, facing onto PC2 (up) and PC3 (right).

3.5.4.3.2 Time Changes These same PCoA plots also reveal that the samples separate out with time, such that the early samples are seen on the left and the later samples on the right of the graph, meaning that time runs roughly left to right in the graph, as can be seen in Figures 3.37 and 3.38. There is some overlap between time points, but in general time appears to separate out the samples.

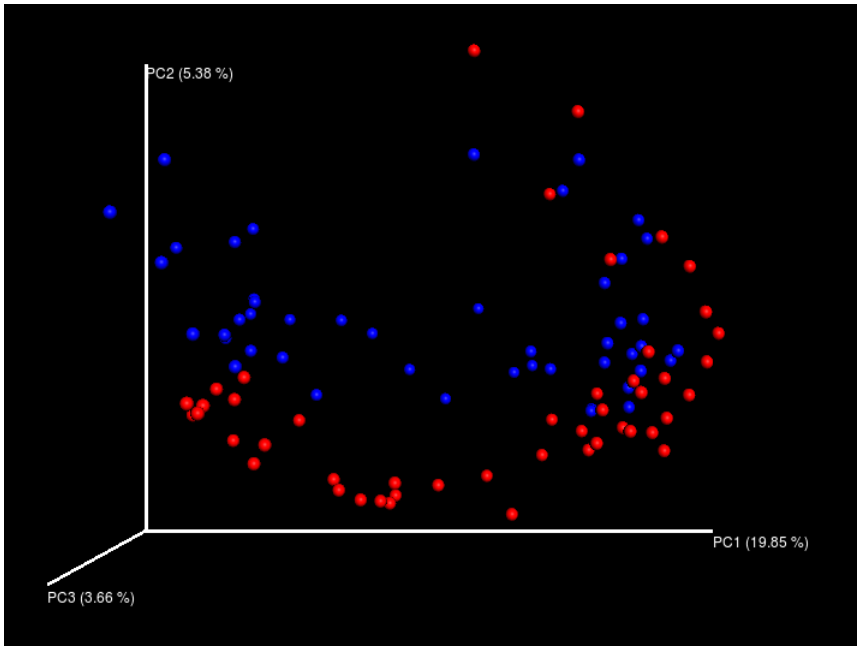


Figure 3.33 *PCoA plot using Unweighted UniFrac. Granite is seen in blue and gabbro in red.*

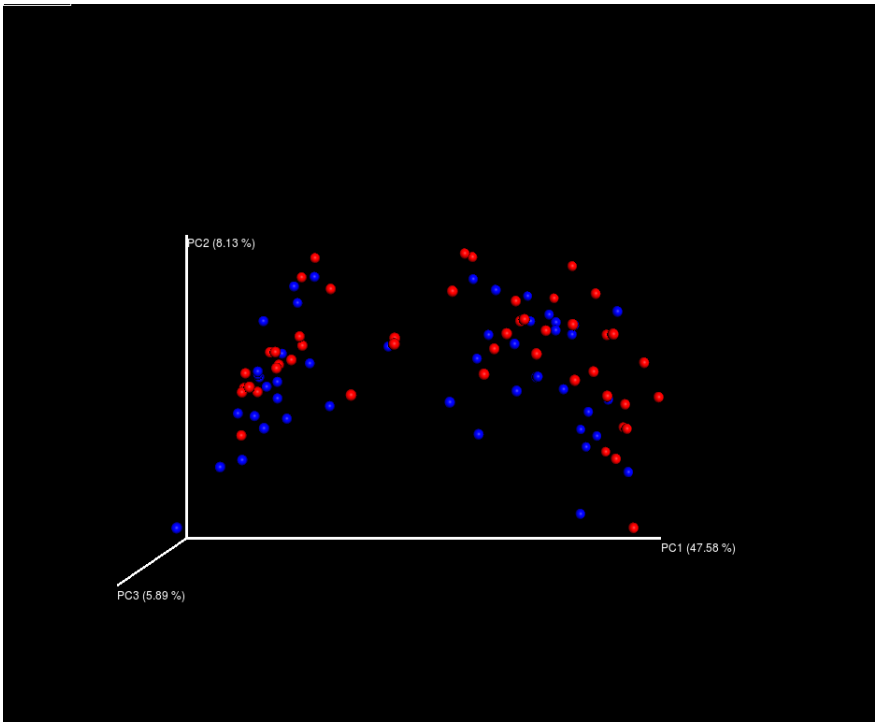


Figure 3.34 *PCoA plot using Weighted UniFrac. Granite is seen in blue and gabbro in red.*

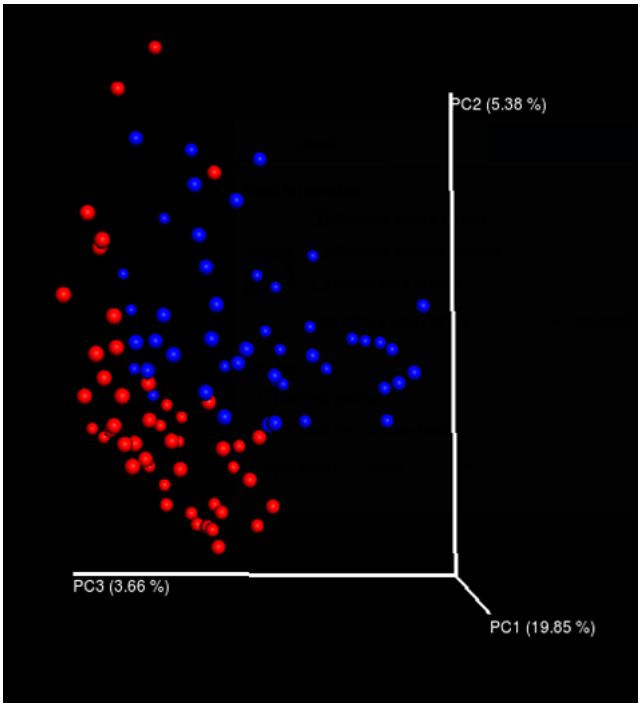


Figure 3.35 *Third dimension of the PCoA plot using Unweighted UniFrac. Granite is seen in blue and gabbro in red. Graphs are viewed from the PC1 axis, facing onto PC2 (up) and PC3 (right)*

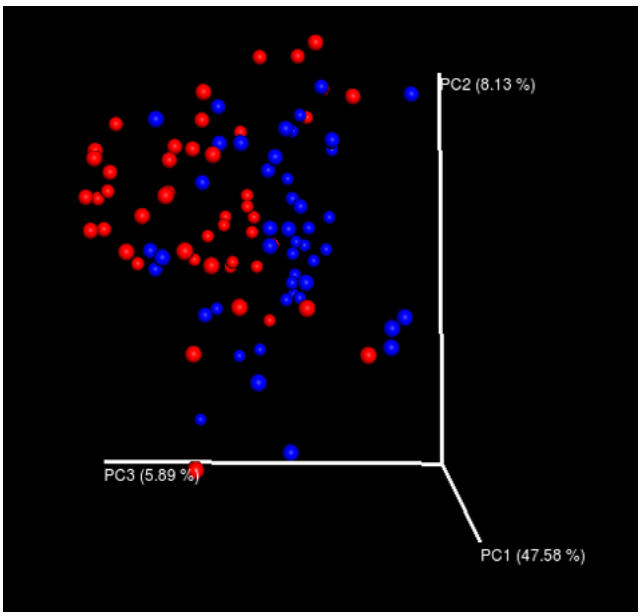


Figure 3.36 *Third dimension of the PCoA plot using Weighted UniFrac. Granite is seen in blue and gabbro in red. Graphs are viewed from the PC1 axis, facing onto PC2 (up) and PC3 (right)*

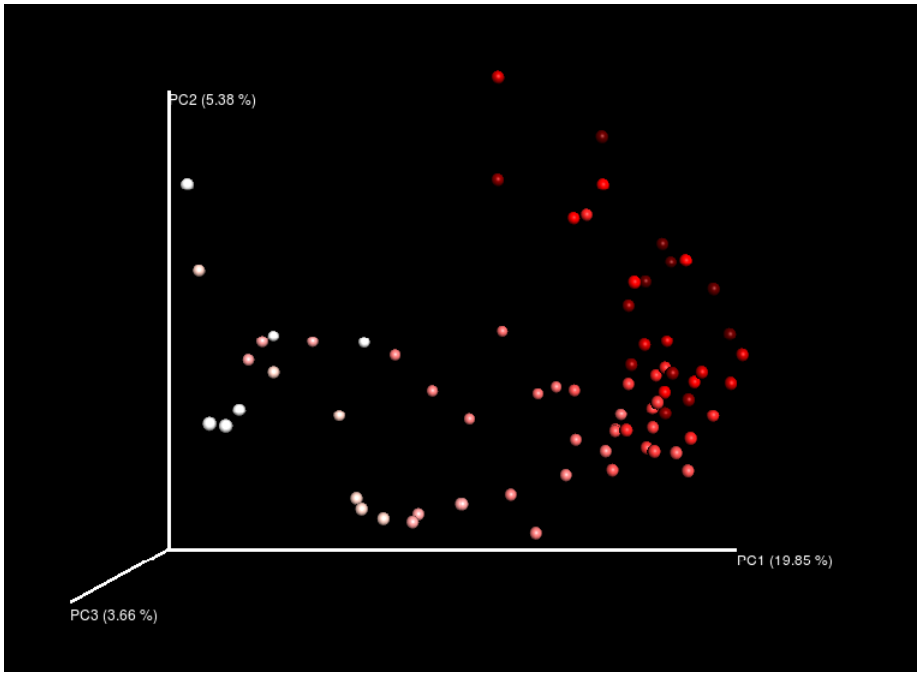


Figure 3.37 *PCoA plot using Unweighted UniFrac. The lighter the colour, the earlier the time point.*

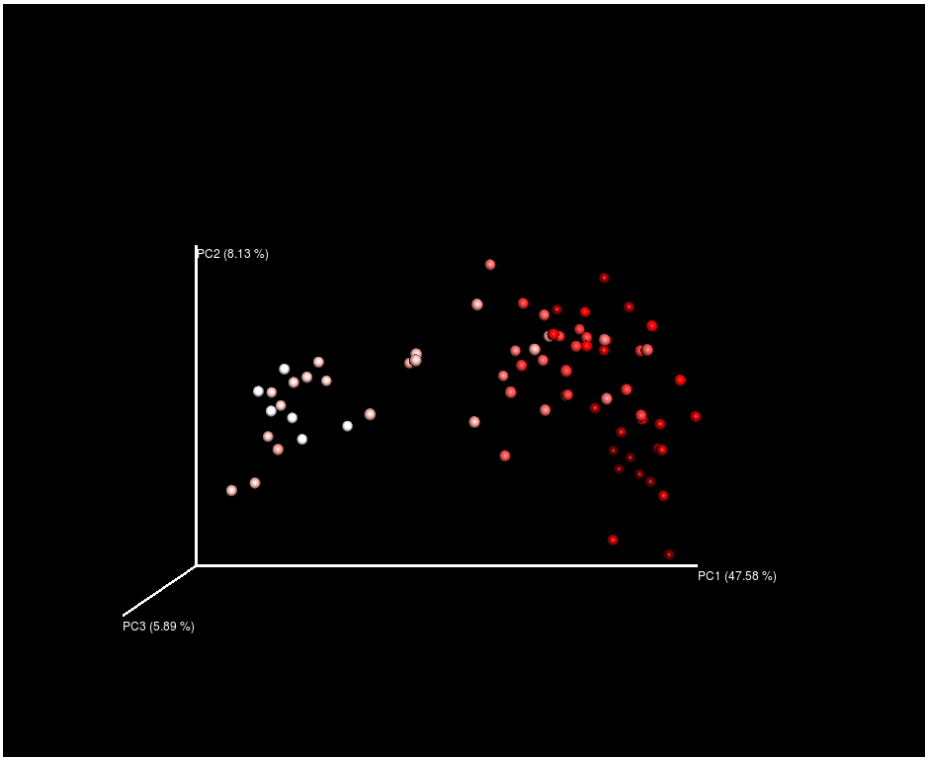


Figure 3.38 *PCoA plot using Weighted UniFrac. The lighter the colour, the earlier the time point.*

3.5.4.3.3 Intermediate Control Samples The PCoA plots also give an insight into the community composition of the intermediate monthly control samples. These are shown to cluster close to the early time points, such that these communities are similar to each other and to Months 1-3 of the actual samples, illustrating that there is little seasonal variation in immigration (Figures 3.39 and 3.40). Note that the last sampling interval between time points 11 and 12 was 3.5 months long, meaning that these intermediate control samples look slightly different from the others, and are seen in the PCoA plots in orange in the middle-right, where they are closest in composition to samples from Months 5 and 6.

3.5.4.3.4 Within-triplicate variation One important feature common to all PCoA plots is that no samples truly overlap, meaning that no two communities are identical, even at exactly identical conditions. This means that for each triplicate of the same rock type at the same time point, the communities are slightly different, such that they separate out on a PCoA graph. For the most part, each set of triplicates cluster close together, but sometimes there is overlap between samples belonging to different triplicate conditions, either by time point or by rock type. Thus, the microbial communities are found to diverge under ostensibly identical environmental conditions. This could be caused either by stochastic processes influencing community assembly or by different inocula on the scale of the microcosms in the experiment, or by a combination of these factors.

3.5.4.4 Stochasticity in community assembly - focus on time points 10 and 12

In order to probe deeper into the question of stochasticity, two time points (time points 10 and 12, representing Months 13 and 18, repsectively) were sampled slightly differently to the others, as described in Section 3.3.11.1. This enabled 9 samples per rock type to be analysed for the influence of stochasticity in assembling the community. The community composition of each of the nine replicates within each condition can be seen at phylum level in Figure 3.41 and at genus level in Figure 3.42. By inspecting the PCOA plots in Figures 3.43 (Unweighted UniFrac) and 3.44 (Weighted UniFrac) it is clear that there is a level of variation between the nine replicate microbial communities within each

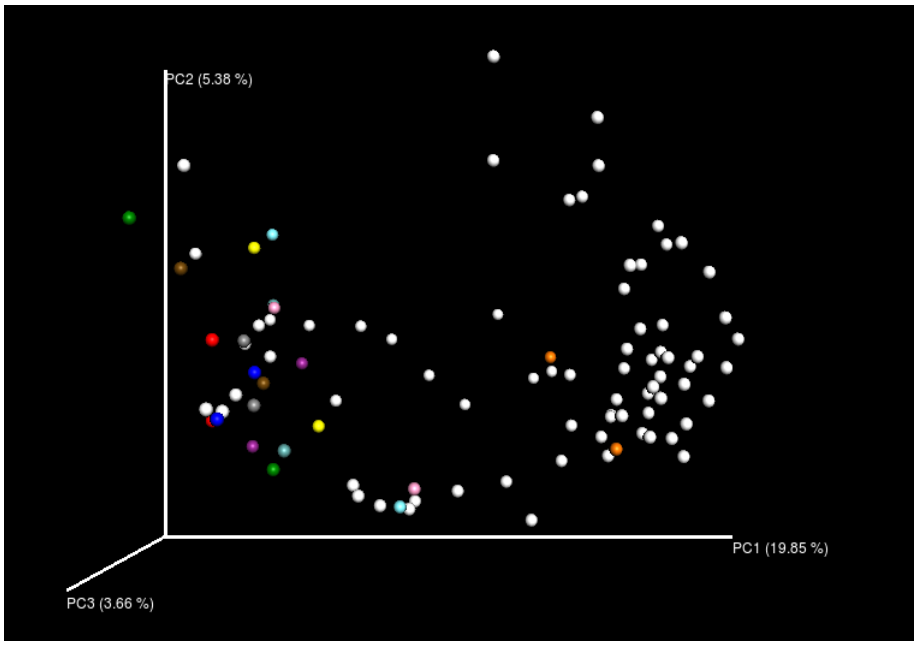


Figure 3.39 *PCoA plot using Unweighted UniFrac for intermediate control samples (colours), with main samples shown in white.*

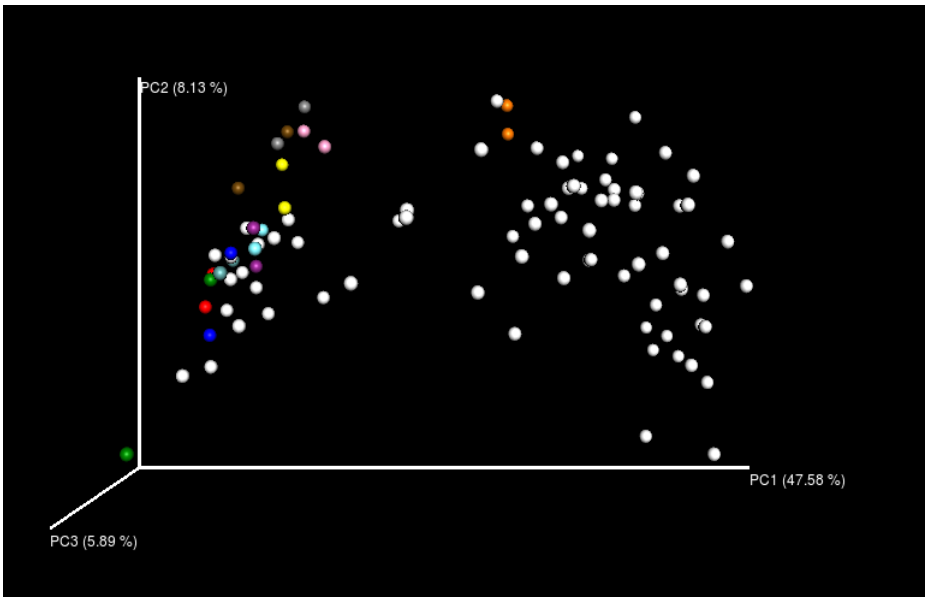


Figure 3.40 *PCoA plot using Weighted UniFrac for intermediate control samples (colours), with main samples shown in white.*

condition. This means that no two communities are the same, even under the exact same environmental and treatment conditions, indicating that community assembly is to some extent influenced by random or stochastic factors. It is observed however that there are broad similarities within each sample condition, such that the community is shaped overall by environmental processes through species sorting, but there is also an element of stochasticity in community assembly, leading to divergence of the communities within each condition.

At phylum level (Figure 3.41), the dominant taxa are Proteobacteria (green), Cyanobacteria (blue) and Bacteroidetes (red). At genus level (Figure 3.42), the dominant taxa is Leptolyngbya (orange), followed by an unidentified genus of the Cytophagaceae family which are environmental bacteria (bottom maroon), an unidentified genus of the Chitinophagaceae family (top maroon), eukaryotic stramenopiles (bottom red) and an unidentified genus of the Bradyrhizobiaceae family (top red). Of note is also the fact that the unassigned proportion in the community is much higher for granite at Month 18 than for the three other conditions, as seen in dark blue at the bottom of both the phylum and genus level graphs.

3.5.4.5 Analysis of generalists

A simple technique was employed to get a crude estimate of the number of generalists in each sample, by recording only those genera that appeared with a non-zero relative abundance in all samples for all time points [87]. Removing all genera that have a value of zero (0) for one or several samples leaves 17 OTUs that are shared between all samples at all time points, and can hence be classified as "generalists" [87]. Of these, the taxa identified down to genus level are *Sphingomonas*, *Kaistobacter*, *Janthinobacterium* and *Pelomonas*. Other OTUs among the 17 shared ones which are not identifiable down to genus level belong to chloroplasts (eukaryotic DNA), Rhizobiales, Chitinophagaceae and Armatimonadaceae.

3.5.5 Analysis of eukaryotic community

It was suspected before the start of the study that eukaryotes may appear in the samples during the course of the experiment. Generally, eukaroytes are not as well adapted to primary colonisation of fresh rock as many prokaryotes are, but

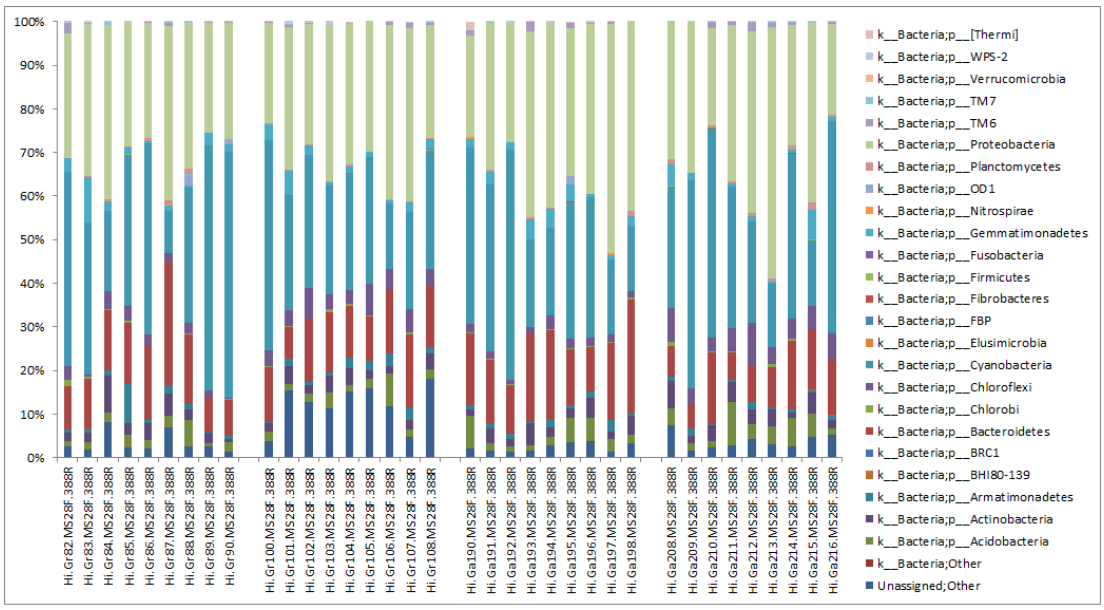


Figure 3.41 *Community composition of individual samples from time points 10 and 12 for granite and gabbro at phylum level. Samples are organised in four groups of nine replicates per condition, showing from L-R: Granite TP10, Granite TP12, Gabbro TP10, Gabbro TP12. Dominant taxa are Proteobacteria (green), Cyanobacteria (blue) and Bacteroidetes (red).*

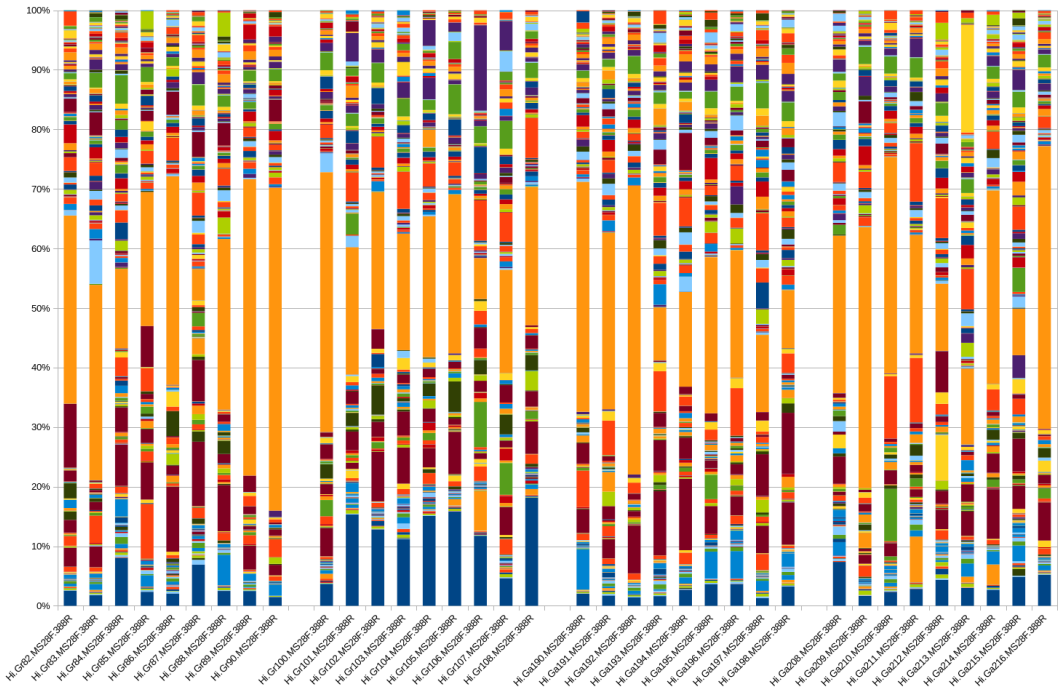


Figure 3.42 *Community composition of individual samples from time points 10 and 12 for granite and gabbro at genus level. Samples are organised in four groups of nine replicates per condition, showing from L-R: Granite TP10, Granite TP12, Gabbro TP10, Gabbro TP12. Dominant taxon is Leptolyngbya (orange).*

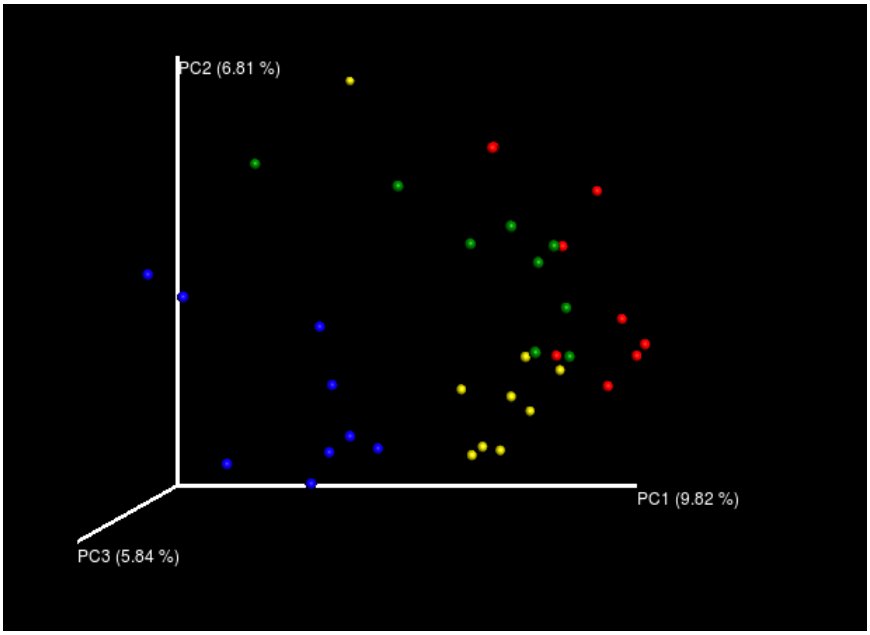


Figure 3.43 *PCoA plot using Unweighted UniFrac for individual samples for time points 10 and 12 Green=granite time point 10, Blue=granite time point 12, Red=gabbro time point 10, Yellow=gabbro time point 12.*

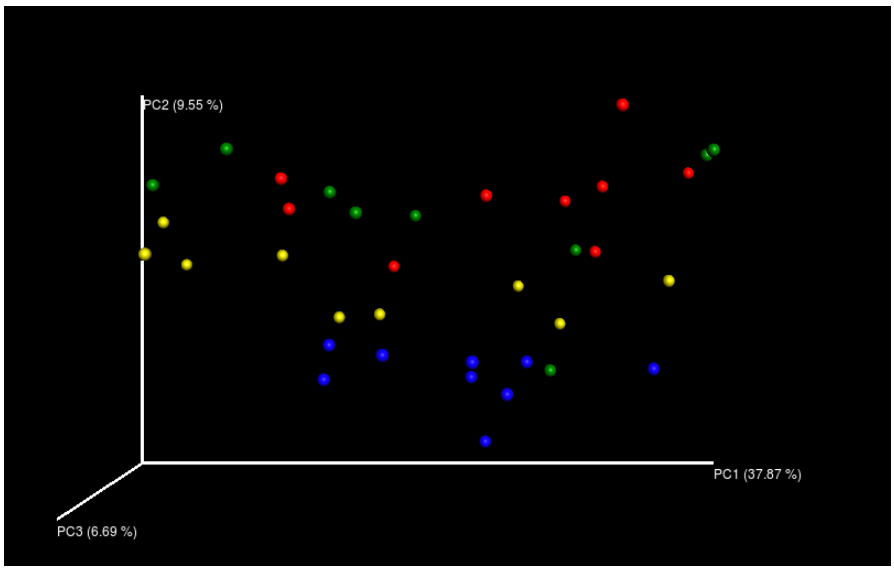


Figure 3.44 *PCoA plot using Weighted UniFrac for individual samples for time points 10 and 12 Green=granite time point 10, Blue=granite time point 12, Red=gabbro time point 10, Yellow=gabbro time point 12.*

it was deemed possible that once a prokaryotic community was established and more nutrients present in the system, it would be possible for eukaryotes to grow, in a similar fashion to the early stages of soil formation. Thus, it was decided to monitor the emergence of eukaryotes in the system, in order to understand when these become a part of the ecosystem in the samples and achieve a more complete understanding of the total community.

The growth of macroscopic organisms, in particular mosses, in some of the samples was observed from Month 3 onwards (Figure 3.45 and 3.46). At Month 3, mosses were only seen in a few isolated samples, whereas their presence continued to increase such that toward the end of the experiment they were visible to the eye in virtually every sample. The eukaryotic community was visualised using microscopy (Figure 3.46) and 18S rRNA sequencing.

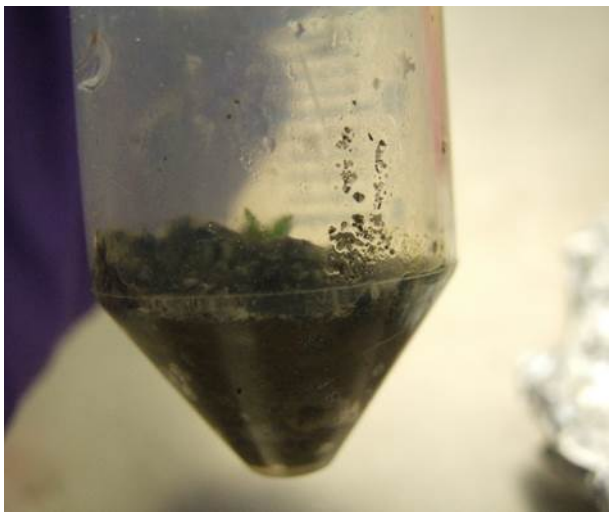


Figure 3.45 *Growth of plants and mosses in the microcosms as seen from Month 3 onwards.*

Samples were sent for 18S rRNA sequencing for all time points. There was not enough eukaryotic biomass to achieve results for either rock type at Month 1 and for granite at Month 2. For subsequent months the biomass had overcome the PCR detection limit, such that the communities could be analysed. For Month 6 the samples were analysed in triplicate in the same way as the 16S analysis, however, for all other time points the triplicates for each rock type were pooled before DNA extraction, PCR and sequencing, such that only one sample was analysed per time point and rock type.

According to the external data processing performed by RTL Genomics (Lubbock, TX, USA), at Month 6, we find distinct differences in the eukaryotic community

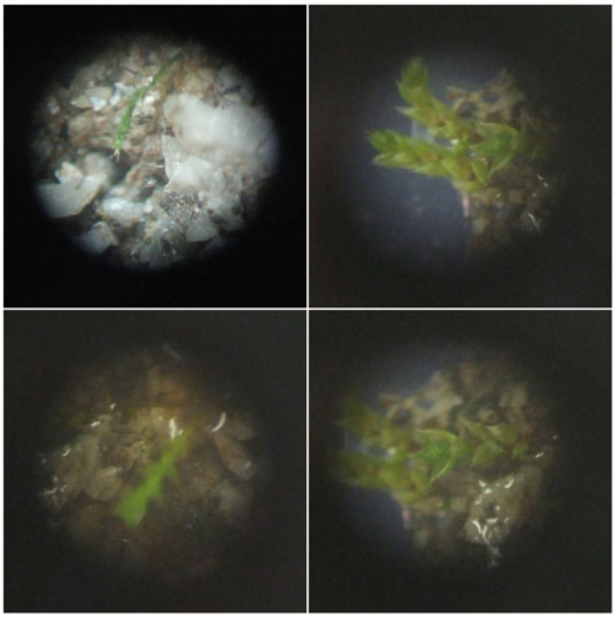


Figure 3.46 *Micrographs of plants and mosses as seen in the microcosms from Month 3 onwards.*

within triplicates, suggesting that the eukaryotic community is more stochastic or opportunistic than the bacterial community. Samples do not cluster strongly to rock type. Some of the most common species are *Bryum sp.* (moss), *Klebsormidium subtilissimum* (green algae) and *Funaria hygrometrica* (bonfire moss). For other months, we also see a large number of sequences corresponding to *Bryum sp.* (moss) and *Klebsormidium subtilissimum* (green algae).

The in-house analysis carried out in Qiime shows the community composition at both phylum (Figure 3.47) and genus level (Figure 3.48). For both rock types at all time points, the communities are dominated by Viridiplantae, which are land plants and green algae. Fungi and Rhizaria (unicellular eukaryotes, made up primarily of the Foraminifera, amoebae, ameoboids and flagellates) are most prominent in the early months, and do not show up to a high relative abundance later on in the experiment. Stramenopiles (heterokonts, primarily algae, ranging in size from diatoms to kelp), Metazoa (Animalia, the animals) and Alveolata (a major group of protists, unicellular eukaryotes) have relatively constant abundances throughout the experiment. Environmental eukaryotic DNA is picked up in the early months, but this proportion decreases over time.

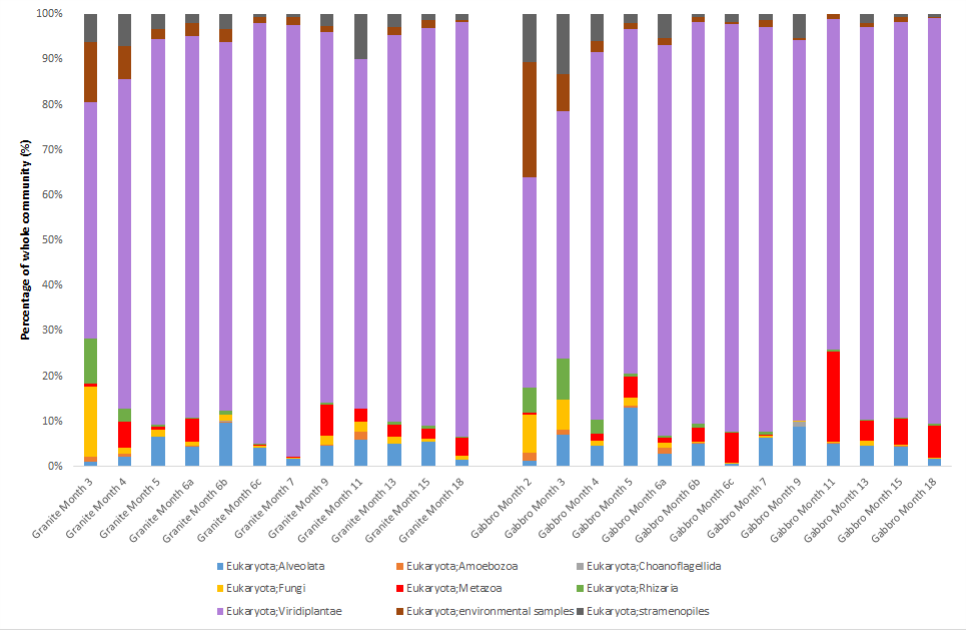


Figure 3.47 *Community composition at phylum level of eukaryotes in the colonisation experiment as seen from Month 2 in gabbro and Month 3 in granite. At Month 6 triplicates were sequenced individually, whereas for other months triplicates were pooled before sequencing.*

3.5.6 Analysis of archaeal community

It was uncertain before the start of the study whether archaea would become established in the community during the timescales involved in this experiment. It was also unclear whether this fraction would be detectable even if present, as it was likely that any archaeal strains present would be slow-growing and few in number. Nonetheless, it was decided to monitor archaeal presence through DNA sequencing, as their presence or absence could not otherwise be determined. Archaea were of interest to the study as they contain several extremophile species that could potentially colonise rock environments such as that studied here, and the decision to include these in the analysis was taken in order to be able to better characterise the overall community.

The presence of archaea was monitored throughout the experiment, by sequencing all DNA samples using archaeal primers. Archaea was only detected in a few samples, as follows: for granite Gr49-51 (Granite Month 6), Gr73-75 (Granite Month 11), Gr106-108 (Granite Month 18), and for gabbro Ga208-210 (Gabbro Month 18) and Ga214-216 (Gabbro Month 18). In addition, archaea was detected in the final granite intermediate samples (Month 15-18), which had only be colonised for 3.5 months. None of these sequences generated identification

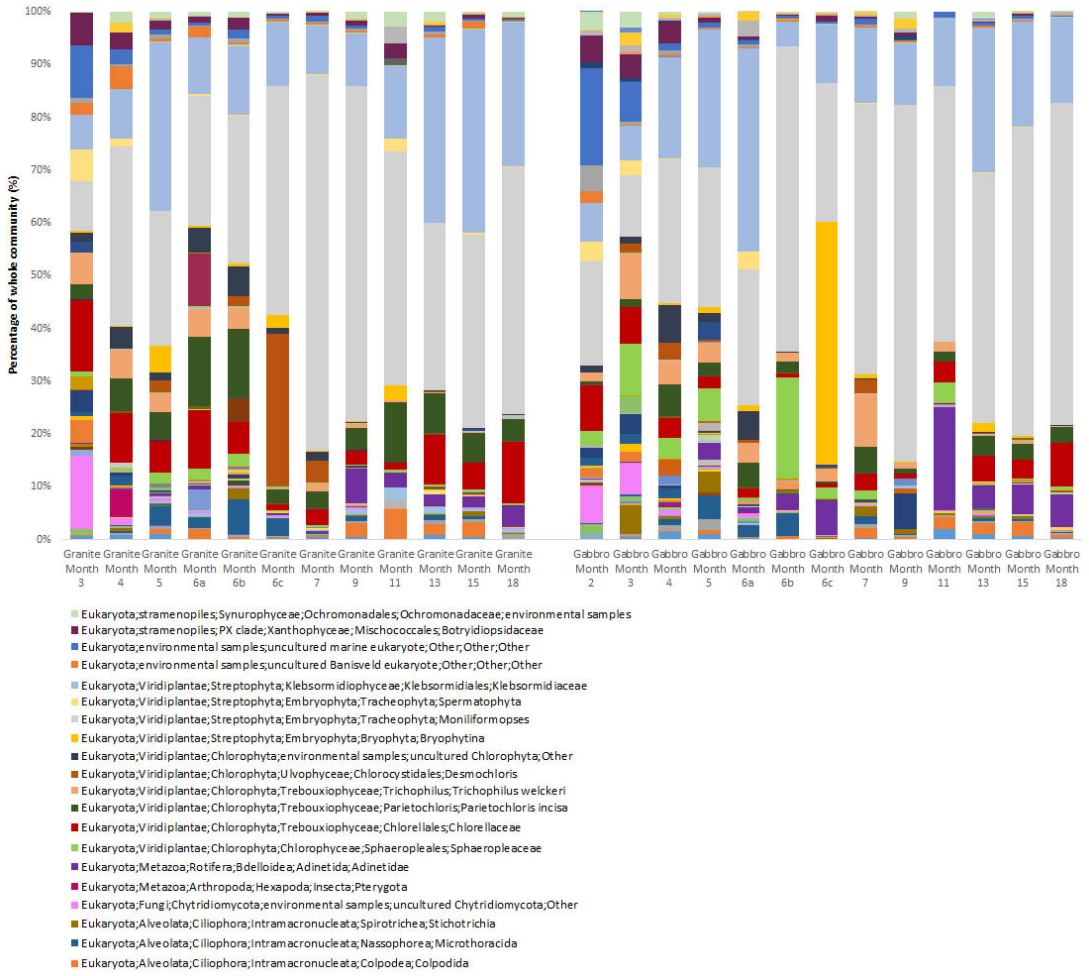


Figure 3.48 *Community composition at genus level of eukaryotes in the colonisation experiment as seen from Month 2 in gabbro and Month 3 in granite. At Month 6 triplicates were sequenced individually, whereas for other months triplicates were pooled before sequencing. Dominant taxa are Klebsormidiaceae (light blue), Moniliformopses (grey), Parietochloris incisa (dark green), Sphaeropleaceae (light green), Chlorellaceae (red) and Bryophyta (yellow).*

through a hit to the database, suggesting that many of these archaeal strains originating from the atmosphere may as yet be unclassified. There is a marked difference in when archaea start appearing between the rock types; for granite archaea is detected at Month 6, whereas for gabbro archaea are not picked up until a year later, at Month 18. Also, archaea is detected in the intermediate granite sample from the final time point, which had only been exposed to the atmosphere for 3.5 months. These results suggest that the granite environment is more suitable for the growth of archaea.

3.5.7 Functional Genetic Analysis

An inferred metagenome was established from the 16S rRNA using the online Galaxy version of PICRUSt [161], with plots generated in Qiime (Figure 3.49). GreenGenes 13.5 [73] was used to create a closed-reference OTU table as the starting point of the analysis. These results must be treated with some caution, as this is only an inferred metagenome, and is likely different from the actual metagenome. According to this analysis, the samples are very similar in their functional composition, suggesting that there is little change in community function over time. If this is true, it suggests that the community gains its full functional capacity rapidly right from the start of the experiment, and that later colonists do not contribute significant new functions as all essential functions are already covered, but that existing functions may merely be performed by different agents. This may explain the variation seen in the communities between identical samples; if the same functions are there and can be carried out by different agents, then variation can naturally occur in the exact species composition of each sample.

3.5.8 Carbon source utilisation

From the carbon source utilisation tests, we find that the gabbro community at Month 6 is able to utilise more carbon sources than the granite community (Figure 3.50). The colours in the figure represent the level of colouring based on a visual inspection. This may mean that the gabbro community is more functionally diverse at this time point and would thus be better able to withstand disruption or changes in the environment. These results must however be treated with caution as this test was designed to identify Gram positive bacteria, whereas it was known after these tests were carried out that most of the community is dominated by

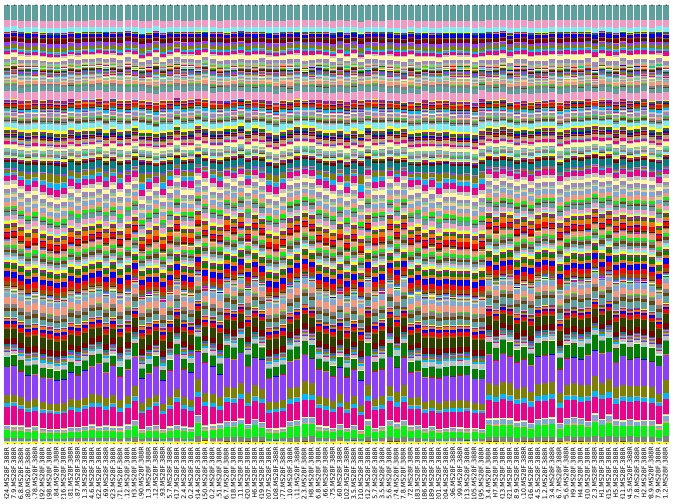


Figure 3.49 *PICRUSt inferred metagenome for all samples (L-R granite Months 1-18, gabbro Months 1-18), suggesting that the community reaches its full functional capacity right from the onset.*

Gram negative taxa. The decision to use these plates as a simple test was because they were readily available in the lab. The composition of the plates in terms of the different carbon sources can be seen in Biolog [28]. The Gram positive plates are still a valid test of carbon source usage, as they were initially designed to identify Gram positive bacteria, but in principle the carbon sources can be used by either Gram negative or Gram positive bacteria presented. The sources used on the two different types of plates are different in that they are adapted to screen for either type of Gram positive or negative strains, and so present carbon sources most commonly used by either Gram positive or Gram negative strains, respectively. There is significant overlap between the two types of microplates and the carbon sources present however, where Gram positive plates are dominated by carbohydrates (n = 41) and carboxylic acids (n = 16), while Gram negative plates contain predominantly carbohydrate (n = 28), carboxylic acids (n = 24), and amino acids (n = 20) [6].

3.5.9 Comparison with an established soil community

Four soil samples were collected and 16S rRNA sequencing performed. Comparing these with the final microcosms from Month 18, there are clear differences (Figure 3.51). The four soil samples are much more similar to each other than to the Month 18 microcosms. The one shared characteristic is approximately

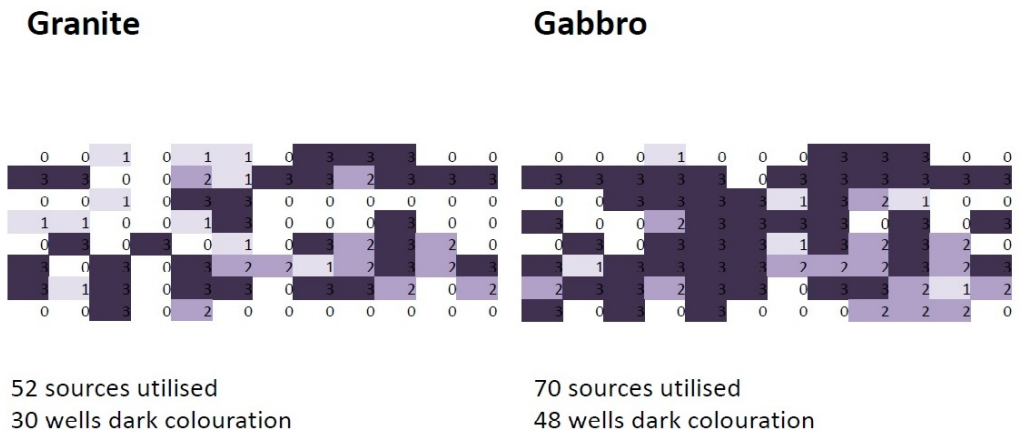


Figure 3.50 *Carbon source utilisation using Biolog plate GP2.*

the same relative amount of Proteobacteria in each sample, around 20-30% in most samples. As previously noted, the two other main taxa at Month 18 are Cyanobacteria and Bacteroidetes, whereas for the soil communities the main phyla are Actinobacteria, Acidobacteria and Chloroflexi. The Blackford Hill samples also see a significant amount of the WPS-2 phylum.

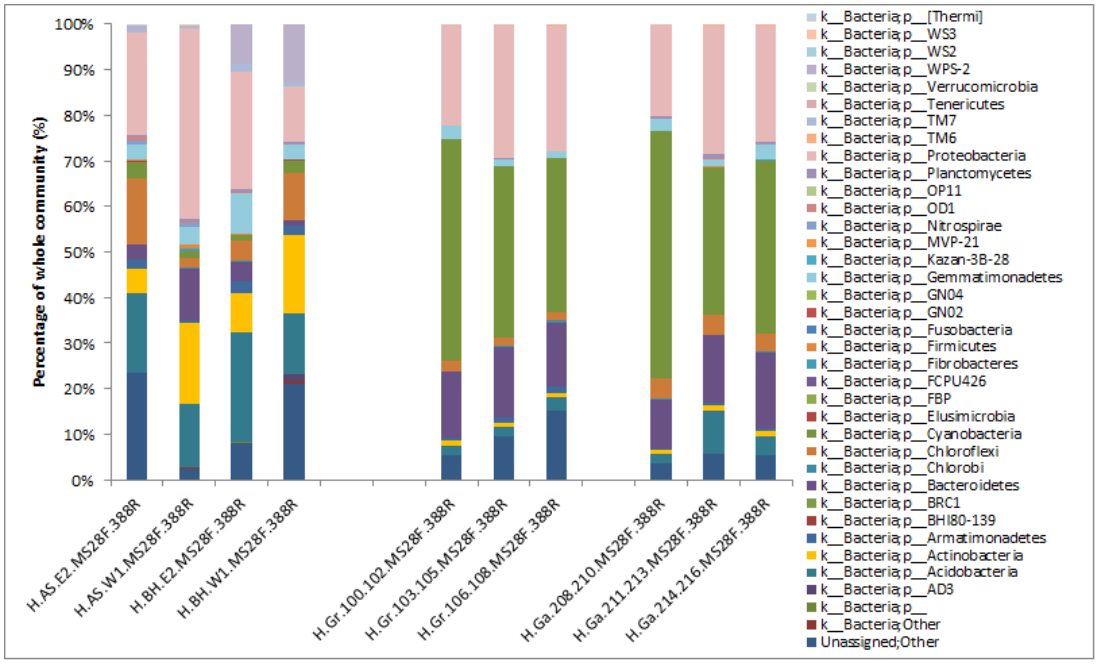


Figure 3.51 *Comparison of 16S rRNA community composition of established soils with Month 18 microcosms. L-R: Two Arthur's Seat samples, two Blackford Hill samples, triplicate granite Month 18, triplicate gabbro Month 18.*

3.5.10 Results summary

In this study, microbial community assembly is influenced by the type of rock substrate and experiences changes over a time period of 18 months. The early community is dominated by Proteobacteria, which are commonly found in similar environments such as fresh lava flows, whereas the later community see a significant increase in Cyanobacteria, which help provide a richer organic environment for the rest of the community. Although the communities are similar between the rock types, there are also differences that make the communities in granite and gabbro distinct.

3.6 Discussion

In this experiment, we have undertaken the first rigorous temporal study of the factors influencing microbial community assembly on a fresh rock substrate. These systems are of great importance in helping us understand how landmasses are colonised, both on the early Earth and at the current time, when fresh lava flows form or after moderate sterilisation events, such as receding glaciers and wildfires. The processes whereby microorganisms interact with rock on large scales are a central part of nutrient cycling and soil formation, and feeds into the global carbon cycle through the release of cations that lead to CO_2 drawdown from the atmosphere. Microbial communities colonising landmasses are integral to many important geological and atmospheric processes, by which both global and local climate stability and nutrient availability are regulated. Through a stringent study where the microbial colonisation of a sterile rock substrate is analysed at controlled intervals, we can better understand how these microbial communities develop over time, and their effect on the rock substrate.

Several unanswered questions concerning how these communities are established and evolve over time were addressed. Previous literature has covered microbial colonisation of igneous environments primarily through single, ad hoc or infrequent time points [145], [147], while rigorous time series of colonisation has been studied in some types of environments, such as artificial endolithic microcosms [53]. No study we are aware of has looked at microbial colonisation over as many time points during the same time period. The microbial communities and succession patterns seen in this study appear to be unique to the environments

studied, but the results correlate with observations from similar studies, on the general trends of microbial colonisation and the types of organisms observed. This experiment was constructed to test specific hypotheses about assembly of microbial communities on new substrates, and thus fill existing gaps in our knowledge about the processes involved in microbial community assembly, particularly related to rocky environments.

3.6.1 Do different rock types select for different microbial communities?

The first question addressed was whether different rock types select for different microbial communities. The results from this experiment show that this is indeed the case - PCoA results showed that granite and gabbro select for similar, but distinct microbial communities. In other words, the environmental selection brought on by the differences in geochemistry in the different rocks is sufficiently strong to impact the developing microbial community. Linking this back to the metacommunity concept, this provides evidence for species sorting playing a part in shaping the microbial community over the timescales involved.

It is important to note that the communities are also very similar between the two rock types. This is likely due to the fact that the geochemical environment might be reasonably similar as the rock types share some common characteristics, and that the microbial inoculum is similar. The microbial community might have been more different on a substrate with less shared characteristics than the two rock types in question.

The communities start out as distinct between the two rock types, but gradually the differences diminish until the communities start to overlap in the later months. This could be explained by the fact that initially the geochemical environments are quite different as they are influenced only by the rock and whatever aeolian input of organics they receive while later on the environments might be more similar. This similarity could arise from the fact that more organics have come in, both through aeolian input as well as dead biomass from the community itself, notably the contribution from algae and Cyanobacteria. Cyanobacteria and algae are considered important ecosystem drivers here as they fix atmospheric carbon through the process of photosynthesis, and making it available to the rest of the community to use as a source of organics. We might thus be seeing more

specialised communities at the onset, while later on the richer environment is able to support a broader community which may be shared between the two rock types.

These findings are in opposition with the hypothesis - namely that the pioneer community would be more specialised and thus similar between the rock types than the end-point community. In reality, the opposite is true, but perhaps for the same reasons - the pioneer communities may be more specialised and hence distinct between the two rock types, whereas the end-point community might be more similar as the environments have become less harsh and allows for a broader community and hence overlap between the two rock types.

These data thus support the idea that the microbial community is indeed adapted to the rock substrate they are colonising, rather than just using it as a surface on which to grow, which could have been a possible interpretation had the microbial communities been indistinguishable between the two rock types.

3.6.2 How does the emerging microbial community change over time during the colonisation of a new rock substrate?

The second question under consideration was how the emerging microbial community would change over time over the first 18 months of colonisation. Here, the early community is dominated by Proteobacteria while the latter has a large contribution from Cyanobacteria. Cyanobacteria are generally slow-growing, and the results here, that Cyanobacteria start to become prevalent around 3 months into the experiment, are perfectly in line with previous knowledge.

In terms of Proteobacteria, the community consists of predominantly Alphaproteobacteria and Betaproteobacteria, which are both well-known colonisers of similar environments such as fresh lava flows [145]. Alphaproteobacteria have been found on Hawaiian volcanic deposits from 1959, while both Alphaproteobacteria and Betaproteobacteria have been found on volcanic deposits at Mount St. Helens at 17 years after an eruption [145]. Alphabacteria are found in a range of environments, notably also on the ocean floor and on sunken whale carcasses, and often are the dominant class in an environmental community where they are found [84], [79]. Both Alpha- and Betaproteobacteria can be found in various

types of soil [79].

Here, for both rock types, the communities are initially dominated by Betaproteobacteria, but this contingent decreases in relative abundance, from about 40% (gabbro) or 50% (granite) at Month 1 to about 10% at Month 18, with the major rapid drop occurring over the first 4-5 months. Betaproteobacteria are often seen as pioneer organisms, and although a very diverse phylum with many different functions, it is a known characteristic that many species are instrumental in nutrient cycling and maintaining soil pH. In addition, several species of Betaproteobacteria are diazotrophs, meaning that they can fix nitrogen from the atmosphere, which is integral to survival for the early colonisers in this experiment and in otherwise organics- and nutrient-poor environments. The presence of diazotrophs is crucial for the survival of the whole community in the earliest stages. The relative abundance of Alphaproteobacteriam fluctuates somewhat between 20-30%, and thus becomes the most dominant class of Proteobacteria in the latter part of the study. Several species of Alphaproteobacteria are phototrophs, which make for successful early colonisers in these types of environments.

Cyanobacteria start out from a negligible relative abundance ($\leq$10%) in the first two months, after which a rapid increase ensues, to a final relative abundance of around 40% in the latter half of the study. The concentration is broadly similar between the two rock types, although some fluctuation occurs. Cyanobacteria are typically slow-growing, usually taking three to four months to become established, which is the pattern seen here. Once they are a significant part of the community, they are an important source of carbon to the rest of the community, by fixing carbon from the atmosphere through photosynthesis.

The relative abundance of Bacteriodetes approximately doubles during the course of the experiment, going from around 8% in Month 1 to about 15% in Month 18. For most of the duration of the study, they are slightly less prevalent in the granite microcosms, whereas both at the beginning and the end the difference between the abundance in the two rock types is negligible. Bacteroidetes are commonly found in the environment, and many taxa are known to degrade organic matter [238].

Thus, the microbial community changes over time during the course of the experiment, and the relative abundance between different taxa changes within the microcosm ecosystems. Betaproteobacteria, Alphaproteobacteria, Cyanobacteria

and Bacteroidetes are all common microorganisms in the environment, and are commonly found in soils and volcanic or rocky environments. Having pioneer species such as Betaproteobacteria and Alphaproteobacteria take up residence in the microcosms early on is consistent with previous findings from studies looking at microbial communities in harsh, rocky environments. Cyanobacteria is found to take a few months before they form a dominant part of the community, which is also consistent with previous findings that these are slower growing organisms that are often present in rocky environments, both on natural outcrops as well as man-made structures such as building facades, statues and gravestones. Bacteriodetes form a smaller part of the community ecosystem throughout, but are also known to be common soil organisms in the environment, and finding that they are a part of these microcosm communities is thus in line with previous knowledge about this taxon. Thus, the changes observed in the dominating taxa at phylum level are consistent with what one may infer from previous studies on similar environments in a natural setting.

3.6.2.1 What are the most dominant taxa at different times of the experiment?

The most abundant genus is the Cyanobacterium *Leptolyngbya*, which make up about 20-30% of the communities in the final time point, and 50% in the microcosm where it is most abundant. *Leptolyngbya* is a very common and widely distributed terrestrial genus with many described species, and is characterised by long thin filaments with narrow cylindrical trichromes of about 0.5-3μm in width. *Leptolyngbya* have a wide range of metabolic capabilities and can be found in many different types of environment and are often associated with soil surfaces [220]. Some species are endolithic and have been considered for use as biofuel and in wastewater treatment due to their capacity for mopping up trace metals and organic contaminants [222]. *Leptolyngbya* have also been found to be part of pathogenic microbial mats that infect corals [187]. In general, *Leptolyngbya* are good colonisers of different environments, and are often found on stone or concrete monuments and even fossilised bone [166], [159], [174]. In some of these studies, *Leptolyngbya* is found to be the dominant genus in the community, as is found in our study. Thus, observing that *Leptolyngbya* is at first a small part of the community but later comes to be a dominant part of the community in terms of relative abundance, means that this is consistent with previous knowledge that these are common environmental organisms found for instance near soil surfaces

or as endoliths, which are similar environments to the ones studies here.

The second most abundant genus is *Janthinobacterium*, which is part of the Betaproteobacteria class (order Burkholderiales, family Oxalobacteraceae). *Janthinobacterium* are Gram-negative soil bacteria that are known for their violet colour, which comes from the pigment violacein and is produced during the oxidation of tryptophan in glycerol metabolism [104]. Certain *Janthinobacterium* species are known and utilised for their anti-fungal, anti-microbial and antioxidant properties [225], [118]. *Janthinobacterium* are known to be extremotolerant, notably of cold temperatures and UV radiation, as well as having broad metabolic capabilities, making them ideal early colonisers that can survive both in the atmosphere and on exposed rock. Here, we find *Janthinobacterium* to be especially prevalent in both granite and gabbro in the early months of colonisation. The presence of *Janthinobacterium* in these communities is consistent with previous knowledge of where these organisms are typically found, and the fact that they are extremotolerant would mean that colonising nutrient-poor rock substrates is within their capabilities, which is observed in this study.

Sphingomonas is the third most abundant genus and belongs to the Alphaproteobacteria. *Sphingomonas* are Gram-negative, non-spore-forming, strictly aerobic bacteria that normally contain yellow or orange pigments and are found in a variety of different environments such as soils, desert sand and lakes [86]. In this study, we find plenty of yellow and orange colonies on yeast extract agar plates, some of which possibly come from the *Sphingomonas* component of the community. The presence of *Sphingomonas* in the ecosystem suggests that these artificial environments are similar enough to natural environments such as soils and desert sands to allow for these organisms to survive.

The fourth most abundant genera is *Pseudomonas*, a Gammaproteobacterium. *Pseudomonas* are defined by being rod-shaped, Gram-negative, flagellated, aerobic, non-spore forming, catalase-positive and oxidase-positive. They are found in a variety of different environments such as soils but also as plant and animal pathogens [226]. Some species are known to be ice-forming and hence perform an important function in the atmosphere in the nucleation of ice and snow [47]. The prevalence in the atmosphere and soils means that it is unsurprising to find abundant *Pseudomonas* is this study. The artificial microcosms thus appear to provide an environment where *Pseudomonas* can thrive and form a part of a wider ecosystem.

Other abundant taxa found are: *Rhodobacter*, a Gram-negative aquatic genus known for their extensive metabolic capabilities, including photosynthesis, chemolithotrophy and aerobic and anaerobic respiration [29]; *Pelomonas*, Gram-negative, rod-shaped, non-spore forming bacteria isolated from haemodialysis and industrial water [112]; *Methylobacterium*, Gram-negative, biofilm-forming organisms that form pink-pigmented colonies and are normally found in soils and water but also as opportunistic hospital pathogens and as DNA extraction kit contaminants [156], [213]; *Mycoplana*, Gram-negative, rod-shaped, filament-forming bacteria [269]; *Flavobacterium*, a Gram-negative genus of the class Bacteroidetes that are found in soils and freshwater [270]; and *Phenylobacterium*, which consists of one known species *Phenylobacterium immobile*, a Gram-negative, strictly aerobic, non-motile bacterium that are only known to grow optimally using artificial compounds such as chloridazon, antipyrin, and pyramidon [80]. Of note is that all the 10 most common taxa in this experiment are found to be Gram-negative.

It is of note that at Month 18, there is a higher amount of Bacteroidetes and Chloroflexi in gabbro than in granite.

The presence of these organisms in the environments studies here suggests that these communities are close to real communities in similar, natural environments. Despite the fact that the communities here are formed in artificially created and sterilised rock microcosms, to the immigrating community these rock substrates could be any newly formed, sterilised or exposed rock faces or environments. The microorganisms that typically colonise these new environments thus appear to be widely distributed in the atmosphere, and can hence colonise new artificial as well as new natural environments easily, with measurable presence of organisms already after one month, and changes in community composition being observed month-by-month for at least 18 months. One concern when setting up this experiment was that placing the microcosms in a city on top of a building may mean that there were mainly human microorganisms present in the immediate atmosphere that would colonise the microcosms. This was found not to be the case - there is very little suggestion that any microorganisms associated with the human population of the city played any significant part in the community. If they were present in higher concentrations in the atmosphere, this did not show up in the community ecosystems on the rocks, as these were dominated by typical microorganisms that are found in soil communities and in rocky and volcanic environments usually not frequented by humans. Thus, the microorganisms that are observed to be good colonisers of new rock environments or frequently found

in soils, such as *Leptolyngbya*, *Janthinobacteria*, *Sphingomonas* and *Pseudomonas* etc., are found to colonise these new rock microcosms in this study, indicating that they are present and widely distributed in the atmosphere in order to colonise new environments, whether that be in isolated environments far removed from civilisation, or within metropolitan areas frequented by people.

3.6.3 How does the immigration into the system change over the year?

The third question being addressed was how the immigration into the system changed throughout the study, and whether there were any noticeable seasonal differences. Figure 3.11 shows that the amount of cells immigrating each month stays relatively constant. From the 16S rRNA results described in Section 3.5.4.3, we see that there appears to be little variation between the intermediate control samples between the different months. These communities are dominated by Proteobacteria (notably *Janthinobacteria*) and *Bacteroidetes*. The communities are similar to each other, and on the PCoA plot there is no noticeable clustering according to the month these samples were collected. We note that the first 7 control samples were collected after 1 month, whereas the next 5 samples were collected every 2 months, and the final interval before the last time point was 3.5 months. There appear to be a little more diversity at the genus level for the longer intervals, especially for the final 3.5 months, where the community appears to be more evolved, which is in line with the fact that this sampling interval is significantly longer than the rest (3.5 months vs. 1 or 2 months). However, there appear to be no seasonal changes in the immigration into the system. This can be related to the weather data obtained, where precipitation and temperature are shown in Figures 3.8 and 3.9 respectively in Section 3.5.1.3. Seasonal variations in temperature are clearly observed, whereas there is little apparent seasonal variation in the amount of rainfall. If microbial input is mostly correlated with rainfall rather than temperature, these observations are consistent with the fact that the microbial immigration does not vary widely over time. There are no previous studies, to our knowledge, with a similar set-up that has analysed microbial colonisation over timescales where seasonality can be taken into account.

3.6.4 Do absolute values of biomass of certain taxa change over time?

It was desirable to monitor the biomass of the microcosms over time. We find that both granite and gabbro sustain about the same amount of biomass, and that the biomass increases until about Month 11, after which the number of cells stay almost constant for the remainder of the study. This is likely due to the fact that population has reached the carrying capacity of the microcosms at this point, as the microcosms are of a fixed size, with limited nutrients that are recycled through the ecosystem. This means that while the community can continue to live and maintain a steady population size, the population cannot increase beyond the means available. The population could decline in the future, if affected by any catastrophic events, but during the period of study, the population size remains steady in the microcosms of both rock types between Months 11 and 18.

The absolute abundance was calculated for the three most abundant taxa at the first few months, *Leptolyngbya* (Figure 3.28), *Janthinobacteria* (Figure 3.26) and *Sphingomonas* (Figure 3.27), utilising the qPCR data combined with the sequencing data giving the relative abundances. These results must be treated with some caution as the qPCR data only takes into account the overall increase in 16S copy numbers or ploidy, and do not account for the fact that many organisms have several copies of the genome per cell, ranging from most organisms being monoploid or diploid up to a known oligo- (between 2 and 10 copies/cell) or polyploidy (above 10 copies/cell) in many prokaryotes, meaning that the qPCR results can be skewed if the average copy number per cell is not consistent over time [283], [198], [32]. It is likely that with a larger, heterogeneous community where most taxa have a low relative abundance and not one or a few taxa dominating the community, this effect from different genome copies per cell would average out over time, but unfortunately, with a complex microbial community this is very difficult to establish with certainty.

It is evident that the absolute abundance deviates slightly from the relative abundance, such that for instance *Sphingomonas* decreases in relative abundance but actually increases slightly in absolute abundance throughout the course of the experiment. For *Janthinobacteria* the absolute and relative abundance profiles are more similar, although the relative abundance is strictly decreasing while the absolute abundance is not. For *Leptolyngbya*, the most abundant genus at the end of the experiment, both the relative and absolute abundance are increasing

during the course of the experiment, and it is evident that there is a large increase between the final two time points in granite, which is a higher absolute increase than the relative abundance shows. For gabbro, the increase is more tempered in absolute numbers than what the relative abundance shows, and the absolute abundance is lower in gabbro than granite at the end. These results show that there is a discrepancy between relative and absolute abundances, such that certain taxa may actually increase in number throughout the course of the study, while their relative abundance can decrease as other taxa proliferate at a higher rate.

There has been a call for more research that reports absolute abundances rather than just monitoring relative abundance, as per a review by Widder et al. [271] (2016) who did not know of any papers in existence at that time that combined high-throughput sequencing data with qPCR estimates of total biomass dynamics over time. Most studies tend to present relative abundances only, and the fact that absolute abundances are not reported is a major aspect lacking in our understanding of microbial community function and structure. As a consequence, an attempt was made in this project to address this gap, and to be able to investigate changes in the absolute abundances of various taxa, and provide a comparison between the changes in relative and absolute abundances over time. The results illustrate that this dual approach is possible and useful, as there are distinct differences between the relative and absolute abundances for the most abundant taxa.

3.6.5 What is the role of species sorting vs. stochasticity in assembling the community?

Support is found for both species sorting and neutral assembly taking place in shaping the community.

Evidence is found for species sorting in the fact that the communities cluster according to the rock type. Even though the communities share many characteristics, there is stronger clustering within each rock type than between the rock types. This means that the environment does select for the resident community in this experiment. Although granite and gabbro are relatively similar rocks, the geochemical environment is different enough to select for the resulting community. Thus, at the largest scale of this experiment, species sorting plays an important role during community assembly.

It is observed that neutral assembly processes also play a role in shaping the community, from the fact that triplicate samples do not cluster in exactly the same place, but show some inherent variation. This point is even better illustrated by the scatter of the nine replicates per condition in time points 10 and 12. Here, again, there is some separation between the four conditions (granite at Month 10, gabbro at Month 10, granite at Month 12 and gabbro at Month 12), such that the communities cluster more strongly within each condition than between the conditions, with some overlap. Within each condition, is observed that when comparing nine replicates, the communities in each microcosm are distinct from the others. Even though all microcosms have been exposed to identical conditions, they do not turn out to host identical microbial communities, but instead display a variety of community compositions. There are broad similarities between the communities, and the communities are more similar under identical conditions than to microcosms under other conditions, such as a different time point or rock type, meaning that species sorting plays a role, and that the environment helps select for the resident community. Yet within each condition they are not identical, indicating a role for stochastic assembly. If the communities were governed solely by species sorting, the communities would be expected to be identical in each microcosm. As the communities show some differences despite all experimental conditions being identical, there is a measure of stochasticity involved in assembling the community. The conclusion is thus that both species sorting and neutral assembly play a role in shaping the microbial communities.

This effect, that microbial communities can diverge under identical conditions, has been observed before. Pagaling et al. [192] studied 100 identical microcosms set up as Winogradsky columns with sediment and a natural inoculum, and found that these resulted in different community trajectories, with alternative stable states developing where different microbial phyla dominate the end-point communities after several months. They found that the communities diverged at a high taxonomic level, with a split seen between communities dominated by either of the phyla Firmicutes or Bacteroidetes.

The colonisation experiment studied here is set up slightly differently, with an unknown component to the colonising community, rather than homogenised sediment. In the colonisation study conducted here, the similarities or differences between the input communities in different samples is unknown, as each microcosm can in principle be colonised by communities with small deviations from one another, if there are variations in aeolian and rainfall input on the scale

of the size of the experiment. It is observed, however, that triplicate samples are closely related, although not identical, suggesting a stochastic element to the community assembly process, if the seeding community can be assumed to be homogeneous. It should also be noted that there is often some overlap between triplicate samples from one time point to another, and sometimes between triplicate samples between the rock types. This experiment supports the conclusion that complex microbial communities can and do diverge under identical conditions due to a stochastic element in community assembly, but are also broadly shaped by environmental factors as per species sorting processes.

3.6.6 Can the emerging microbial community composition be linked to different environmental variables, such as temperature and pH?

One of the questions to be addressed was whether microbial community composition could be linked to any changes in the different environmental variables present. One difficulty is that some environmental variables are not easy to measure, and some responses are likely due to an interaction of variables and feedback within the community itself. Of the variables measured, pH and elemental leaching likely play the largest role, and are both influenced by the rock substrate, meaning that the difference between the two rock types can manifest itself as different geochemical environments, as measured by pH and elemental leaching. As such, pH and elemental leaching are observable variables that, if found to shape the community, would support species sorting as a community assembly mechanism. These variables can also change over time, meaning that they can affect the community continuously throughout the experiment.

Changes in the pH likely affect the community composition over time. Changes in pH are caused by the rock geochemistry and the acids generated by microorganisms through their metabolism. Initially, the pH of granite is about 8.0 and for gabbro this is about 9.5. Over the course of the experiment, we see both of these drop to about 7-7.5, with gabbro providing an almost consistently higher pH, apart from at Month 11. It is unclear whether the pH has settled toward the end of the experiment, or whether it would continue to fluctuate or decrease if the experiment had been extended. The fact that granite generates a more acidic environment is consistent with it being a more acidic rock, due to its higher silica content. In terms of the values that pH can take, the changes

seen here are smaller than they might be in a closed microcosm, where the pH could easily drop to 3.0 or lower due to a net build-up of acid as a byproduct of microbial metabolism. This was prevented here as a flow-through system was adopted, such that it would be difficult for acid to build up to significant levels. However, given that pH is a logarithmic scale, even the changes seen here might be significant enough to affect a change in the community if it drops below the optimal or minimum growth ranges of some of the species in the community. pH is likely one of the major variables that control the microbial community in this experiment, its decline corresponding with changes in the community composition. pH values are more similar between the two rock types at the end of the experiment, suggesting that there is likely to be a higher growth range overlap between the communities towards the end. This is in keeping with the community composition changes observed from the betadiversity analysis - that the communities appear to converge, such that the differences between the communities in the two rock types are attenuated over time. The impact of pH and an altered geochemistry on the communities supports the theory of species sorting as a community assembly mechanism, as the environment appears to have a hand in shaping the communities.

Temperature is thought to have some influence on the community, however, seasonal variations in ambient temperature at the sample location is relatively small, as can be seen in Figure 3.9. One can imagine that if the experiment had been performed in an environment with a larger temperature range and longer periods of extreme heat or extreme cold, the community might have seen bigger changes in composition. The weather data in Figure 3.9 shows that temperature does fluctuate during the course of the experiment, and that there are 48 days at which freezing occurs during the experiment. Most organisms that can live under the conditions present in the experiment will be adapted to some changes in temperature and repeated freeze-thaw cycles (for more investigation on this topic, see Chapter 4). If temperature has an effect on the community, these changes would be observed over longer time scales, and likely occur seasonally with the heating and cooling that occurs through a year in temperate climates. These patterns may have been easier to distinguish if the experiment had ran for several years, where seasonality could be monitored over several cycles. Here, only six months of the year were repeated throughout the experiment (Month 12-18 being the same time of year as Month 1-6). Still, it is likely that various temperature events did shape the community at a scale that could not be made out, and one might need finer sampling intervals in order to capture transient population

crashes after for instance a freezing event. Equally, the overall conclusion from the study here is that seasonal variations in temperature does not appear to majorly impact the community on the larger scales.

3.6.7 How does the elemental leaching change throughout the experiment?

Elemental leaching is key to establishing the geochemical environment in the microcosms in this experiment. Here, elemental leaching was measured at different time points in order to establish whether the geochemical environment changed over the course of the experiment. This is found to be the case, and the end-point communities experience a different environment from that of the first colonisers, which can be correlated with some of the changes in the microbial community seen over time. For both rock types, we see an increase in the leaching of Mn, Ni and Zn, and a decrease in Ti and Na. For granite, we also see an increase in Fe and Cu and a decrease in K, Ca, Mg and S. For gabbro, we see an additional increase in Al and Fe.

It is possible that the microbial community in granite utilises more Fe towards the end of the experiment, whereas the reverse would be true for gabbro, as the concentration of Fe increases in the granite microcosms but decreases in the gabbro microcosms during the course of the study. Both Co, Cu and Zn increases throughout the experiment for granite while for gabbro the increase is much smaller, meaning that these elements are more readily available to the community in granite than in gabbro. The presence of Ti appears more or less constant for both rock types until 12 months, but decreases sharply between months 12 and 18. The concentrations of Mn and Ni increase during the experiment for both rock types. Al is present to a relatively high concentration only in gabbro at the outset, while it decreases over time to similar concentrations as observed in granite throughout the course of the experiment. S is only present in granite at the start and then decreases rapidly between Months 1 and 6, while it is present in only negligible amounts in gabbro throughout the experiment. The amount of Na decreases steadily in both rock types, while for Mg and K this is only observed for granite, while the gabbro levels stay more or less constant. Some elements follow the same leaching trajectory, notably K and Mg having the same profiles as each other and Co and Cu having the same profiles as each other. These results show that the weathering profiles are distinct for granite and gabbro throughout

the course of the study.

It is found that there are significant differences between the rock types for more elements at the beginning than at the end of the experiment, indicating that the environments become more similar between the rock types over time. This correlates with observations that the microbial communities are becoming more similar over time and start overlapping, which could be due to the environments becoming more similar. Thus, there may be a link between the changes seen in the overall chemical environment and the changes seen in the microbial community from the start to the end of the experiment, and the fact that the geochemistry is becoming more similar may lead the communities onto similar trajectories, reducing the differences in community structure.

Relating the presence and changes of certain ions with presence and changes of certain microbial species present is an interesting question, albeit not one that it is possible to answer directly with the level of data gathered here. As samples were measured and analysed in bulk and many elements exist only in low concentrations, it is not known how elemental concentrations vary on microbial scales throughout the samples. Further work and new techniques are required in order to better be able to draw these parallels to understand the specific effects of certain ions on metabolism in a community bulk setting.

3.6.8 Other results

3.6.8.1 Comparison with an established soil community

It is clear that the end-point communities in this experiment are quite dissimilar to an established soil community after 18 months. The community in the soils is dominated by mostly other phyla than the microcosms, although the Proteobacteria fraction is shared. Thus, the length of time that this experiment ran for is not enough to approach a real soil community. There are a number of reasons why that might be the case.

Firstly, the soils sampled all come from areas with a high exposure from and cover of macroorganisms, such as plants, fungi and animals, and contain abundant organic substances. Although the scree slopes that were sampled were barren of vegetation, they had significant plant cover all around, and in some sites rabbit droppings were visible in the general area. This is in contrast to the microcosms,

which had little influence of plants and animals, other than a few leaves and bugs that found their way into some isolated microcosms at various time points. Any macroscopic foreign bodies, such as bugs and leaves, were removed one-by-one in a sterile manner from each sample at each timepoint before any treatment or analysis was instigated. It is possible that these items have brought in microorganisms to the system that would not have been dispersed through the air, and that these may thus skew the results from what they may have been if no macroscopic particles had entered the system. Several different mitigation strategies were considered in order to have potentially avoided this confounding variables, however, none of the proposed systems were deemed appropriate as they would bring in other concerns. For instance, using a mesh cover would likely have slowed down or inhibited immigration into the system, as water droplets may have been caught on the mesh through capillary action. Ultimately, it was decided that using an open system would most accurately reflect how colonisation takes place in real environments on Earth today, and was thus the preferred choice.

Secondly, the extensive growth of Cyanobacteria and algae into the microcosms is in contrast to the much smaller relative abundances of Cyanobacteria seen in the normal soil communities that were analysed in this study as a comparison. Instead, the amount of Cyanobacteria is more similar to communities that are found on exposed rock surfaces on coastlines or in other environments rich in moisture, or even rock monuments, houses or walls. Cyanobacteria are frequently found on walls where water is present, such as around a leaking pipe. It is likely that the microcosm design here created a good, moist environment for Cyanobacteria and algae to grow, as the tubes provide shelter and delayed the rocks in drying out after rainfall.

Thirdly, the soil communities were growing on substrates that are different from those in the experiment. In the experiment, we utilised crushed up rock of two specific types, which were not mixed. The soils were collected from scree slopes of rock that are somewhere between granite and gabbro in composition. In addition, the rock substrate in the soil samples is likely to be a mixture of rock types and soil types due to turnover, hence providing a different and richer environment than that of the microcosms.

These factors combined likely led to the fact that the microcosm communities are different from established soil communities. It is unclear from this data whether the community trajectory would have led closer to a soil community had the experiment continued, and if so, what the timescales involved would be.

3.6.9 The emergence of phototrophs, archaea and eukaryotes

Although the colonisation of this novel environment by bacteria was the focus of this experiment, it was recognised that it was very likely that colonisation by phototrophs, archaea and eukaryotes would also potentially be observed. In order to fully be able to characterise the community, it was deemed important to also screen the samples for the presence of these other taxa. The presence of these other taxa in the samples may have an effect on the bacterial fraction, in that they may drive changes in the composition of the bacterial community due to competition for space and nutrients, while at the same time potentially making new types of nutrients available for the bacterial community to utilise, such as phototrophs providing further carbon sources.

Phototrophs do not appear in significant amounts in the DNA sequencing results until Month 3, but it is evident that they are in the system already at Month 1, as shown by the phototroph culturing. As most phototrophs are slow growers, it is no surprise that they are not present in significant numbers at Month 1 in the sequencing.

Archaea were only present in a few samples analysed, and did not form a significant part of the final community.

Eukaryotes were observed in all samples from Month 3 onwards, and it was possible to analyse the community composition through the sequencing analysis performed. The eukaryotic community is found to change over time, which is similar to the results observed for the bacterial fraction, and it is found that the eukaryotic community is more heterogeneous than the bacterial community within identical triplicate samples, indicating that the eukaryotes may be more stochastic or opportunistic than the bacterial community.

3.7 Limitations

There are several potential limitations to this study, most of which could only have been overcome by compromising at other points in the experimental design. Future studies can enhance the field by addressing these limitations.

3.7.1 Edge effects

One potential limitation in this experiment is that the sample box had relatively high sides of about 50cm. This means that potentially there was an edge effect whereby samples close to the edges received less rainwater and thus less net influx of colonisers and nutrients. Any future similar studies should try to minimise the height of the sides of the box. Here however, the samples were arranged in a random grid in the box, meaning that over the course of the experiment, any effects due to sample location in the box should cancel or even out. Also, there is no evidence from this experiment that suggest that the samples near the edges were any different from the samples closer to the middle.

3.7.2 Sample temporary relocation

For about two months in the middle of the experiment, the whole sample set had to be moved about 20m from its original location before being returned there. This was due to essential estates maintenance that had to be carried out, namely relaying of the roof on top of the James Clerk Maxwell Building where the samples were sitting. In order to accommodate this, the sample box was carefully moved to a new location on the same roof, roughly 20m away from its original location for the duration of the roof works. During this time, the samples experienced minimal human contact as the new location of the box was well away from the area where the work was carried out. Once the work was finished, the box was carefully placed back at its original location, after which it remained there for the rest of the experiment.

The results from the experiment show no unexpected deviations during this time, indicating that the temporary relocation had minimal impact on the data. This also indicates that colonisation is homogeneous on a homogeneous surface over distances on at least the order of 10s of metres.

3.7.3 Possible effects of placing samples at height

It is possible that placing the samples at height, in this case at the top of a building, meant that the aeolian input of colonising organisms were not the same as what they may have been at ground level. At ground level, especially in close

proximity to soils or overhanging vegetation, it is possible that the microbial input may have been different due to convection, however, there are no studies to our knowledge that helps assess this situation accurately. In this instance, the samples were placed on the ninth floor on the roof of a building, placing them about 100 ft above ground level. The reason for this choice was to limit tampering with the samples by animals or humans, and placing them on the roof meant that there was more restricted access by macrofauna to the samples than there would have been if samples had been placed somewhere on the ground.

3.7.4 Substrate similarities

An aspect of this study to consider is that the two substrates under investigation are fundamentally quite similar. Both granite and gabbro are intrusive, igneous rock types that share many of the same bioessential nutrients, even though their mineralogy is different. A different approach to the study might have been to select two vastly different substrates, or even two very distinct types of rock, which may have led to the observation of stronger species sorting effects. The reason for choosing these two rocks was that they make up the majority of the Earth's crust, and are by nature relatively similar rocks. The conclusions drawn from this study is that the Earth's surface is a fairly homogeneous environment from the point of view of a microorganism. The differences between the two rock substrates that constitute the majority of the crust are not so large as to drive distinct differences in the microbial community structure. That being said, this experiment tested the two rock types under otherwise identical conditions, whereas on the present Earth these parts of the crust experience very different environmental circumstances, the gabbro crust being found mostly under the oceans, while the granitic crust form the continents.

Another possibility that was considered was to include a third substrate that might provide a very different environment, however, this was decided against due to the sheer scale of the experiment and number of samples involved. We instead opted for a rigorous experimental set-up with two rock types and as large a number of samples and time points as possible to address the questions at hand. Even so, the experiment consisted of 264 individual microcosms, which took several weeks to set up and prepare, and each sampling point took over a week of full working days for the one experimenter to collect and analyse. Thus, choosing to work with granite and gabbro ensured that the experiment stayed at

a manageable level as well as suiting the remit of the study in order to be able to address the chosen hypotheses.

3.7.5 Geographic limitations

The fact that this experiment was conducted in only one geographic location is not necessarily a limitation, as we did not address any questions specifically related to biogeography. However, the experimental set-up could easily be employed to test these types of questions if it were repeated in several locations. This could be done in order to test the "everything is everywhere" hypothesis [13], on both smaller local scales as well as intercontinental scales. The same rigorous experimental set-up would be employed at the various sites, and could give insight into how colonisation and community assembly might vary with location.

3.7.6 Soil community comparisons

The soil communities that were analysed here as a comparison with the end-point 18 months samples were not perfect analogues for the experiment microcosms. However, there are other soils that would serve as a better comparison, such as pristine basaltic or granitic soils found for instance in Iceland. For this experiment it was decided to sample local soils, as the global distribution and variation in atmospheric microbial content is not well understood, and hence we chose to sample soils near the site of the experiment.

3.7.7 The influence of eukaryotes

The fact that eukaryotes such as algae and mosses ended up taking up a big part of the biological community in this experiment was not something that was taken into account when the experiment was planned. However, it is not surprising to see mosses and algae in these sheltered, most rocky environments, which provided good growing conditions. The eukaryotic contingent likely had an impact on the accompanying bacterial community, such that in the absence of eukaryotes the bacterial community might have looked very different. For future experiments, one could consider covering the microcosms with a mesh that would stop smaller seeds from getting through, however, it is difficult to know whether this would

also not block out much of the bacterial immigration either by water droplets or other particles carrying bacteria not getting through.

3.7.8 Bias concerns from sequencing analysis

DNA-based studies come with some inherent biases and limitations.

Firstly, there is always some degree of extraction bias, as it is not possible to ensure that all DNA present in the sample ends up being extracted and thus present in the final analysis. In this instance, it is likely that cells that were more tightly bound to the rock would have been less efficiently extracted. Additionally, some cells are easier to break up and efficiently extract the DNA from than from other organisms.

Secondly, the community profile that is attained through sequencing is often not completely representative of the viable community present in the samples, although the viable community is likely to be a large subset of the sequenced community. This is due to the fact that a sequencing analysis will pick up also DNA that is either environmental, or part of organisms that have drifted into the sample but are not active within the community. In order to characterise the viable community, other techniques such as culturing must be employed.

Thirdly, there are a number of biases that can be introduced in the analysis at the PCR stage. For instance, the genomes may not be amplified evenly across all organisms, such that PCR may enhance the presence of certain organisms in a sample, which then appear over-represented compared to the original ratios in the sample [140]. Additionally, there exist in each PCR reaction a certain degree of base substitution, whereby the wrong nucleobase is added to a sequence, which has implications for subsequent correct species identification. The formation of chimeras is another problem, whereby during PCR an amplicon isn't added properly, and during the next cycle it instead acts as a primer and can bind an unrelated amplicon, which means that two different templates have merged into one sequence. These sequences are artifacts introduced during PCR, and, although there are steps taking during the data analysis to identify these, they can go undetected and thus appear as novel species in the final analysis.

There are similarly errors that can be introduced during the sequencing step itself. During sequencing, a fluorescently labelled base is added to the single

strand, and there is a possibility that the wrong nucleobase is bound at each step, as all nucleobases are present in the mix. This leads to the possible creation of false sequences, and there are steps during the data analysis where this effect is reduced, such as having a cut-off Phred score, where the Phred score indicates the certainty that the correct base has been identified.

Additionally, there are biases or artifacts that can be introduced during the data analysis steps. For instance, there is a certain confidence level associated with OTU clustering, such that it is possible to cluster sequences together that do not actually belong together. Typically, a threshold of similarity is chosen, and sequences above this threshold grouped together as one OTU, however, this is a somewhat arbitrary choice based on current community standards, and a chosen OTU may not represent a single species. Due to the problems associated with sequencing errors and the creation of chimeras, false OTUs may be created that do not reflect a real species, and thus the diversity of a sample can be overestimated.

3.8 Conclusions

This experiment has deepened our understanding of the emergence of complexity in pioneer microbial communities. The communities are found to be similar but distinct between the two rock types. The communities appear to be converging throughout the course of the experiment, which may be a result of the environments becoming more similar. We find that both species sorting and neutral assembly processes influence the community assembly on different scales. We find succession in outdoor rock microcosms over a period of 18 months, despite the immigration remaining unchanged. This experiment is the first controlled time series study of the microbial colonisation of rocky environments, leading to new insights into how complexity in these communities emerges.

Chapter 4

The impact of environmental stress at different stages of community assembly

Microbial community assembly is a complex process where multiple factors, such as environment, dispersal and historicity play a role [189], [191]. Taken together, these factors help shape the structure of the emerging community as it increases in complexity. Specifically, different types of environmental stress can influence the community by selecting for certain taxa over others. Environmental stressors that can impact an emerging community are changes in, for instance, temperature, pH, salinity and availability of water and nutrients [52]. Currently we have a limited understanding of the time course of community formation, and the role of environmental perturbation at different stages of assembly. An experiment was set up in order to study the impact of environmental stress during different times of community assembly, comparing the stress responses of samples that were 2 and 14 months old. It is hypothesised that the early community would be better at withstanding environmental stress as it mainly consists of generalists that are good colonisers and able to survive in a range of conditions, while the older, more complex community is likely to be more adapted to the particular conditions present in its habitat [145]. Here, stress was tested in the form of freeze-thaw cycles, desiccation cycles and pH alteration, which are environmental stresses commonly experienced by rock-dwelling communities. The results indicate that the younger community is better at withstanding stress than the older community, potentially because the early community is at 2 months made up of early colonists

that are adapted to survive harsh environmental fluctuations.

4.1 Introduction

Ecological disturbance, where an ecosystem is subjected to external changes in environmental conditions, such as temperature changes, desiccation or nutrient loss, is of global concern as fewer and fewer areas remain unperturbed by a changing climate and increased human activity. The success of a particular ecosystem experiencing alternating environmental parameters relies on its ability to adapt to these conditions without biomass or functional losses. Among the most versatile organisms on the planet are microorganisms, such as many bacteria and archaea. Microbial ecosystems thus contain a multitude of functions and are often able to thrive under a vast range of environmental conditions. In the process of colonisation, new ecosystems are put together in a novel habitat, where environmental conditions can be fluctuating and challenging. These fluctuations can create environmental stress on the emerging community, resulting in changes in community composition or entire population crashes [157]. In order to become established, an ecosystem needs to be able to survive and adapt throughout the process of colonisation. Evidence has suggested that a young community consisting only of a few species, may be more susceptible to perturbations, and that biodiversity may act as a buffer against external stress [218], [14]. However, our understanding of whether ecosystems are equally resistant to stress at all times during colonisation is currently limited.

In biology, pioneer species colonising new habitats tend to be versatile and adaptable in the parameters required for survival. Early colonists are thus known for being vigorous in the face of changing or adverse environmental conditions [164]. As such, they tend to have a wide growth range in terms of nutrient use and of environmental parameters such as temperature and pH, as well as being able to withstand freezing and desiccation. These characteristics allow pioneer species to colonise a range of emerging habitats, provided that adequate dispersal mechanisms are in place, while more specialised species can only colonise those habitats that fulfill their requirements. On the scale of microorganisms, a new, virtually sterile habitat is often rocky, exposed and nutrient-poor [67]. Some examples of new habitats open for colonisation include, from most sterile and nutrient-poor to less sterile and more fertile: fresh lava flows, glacial forefields and forests after wildfires.

From other work forming a part of this thesis, it was observed that complexity of community composition and diversity increased over time as communities matured (see Chapter 3). Thus, the younger community has a lower diversity than the older community. In other scenarios it is possible to have low-diversity mature communities, if there are limitations on for instance nutrient availability or immigration into or emigration out of the system. In these instances, a closed-loop, low-diversity ecosystem may develop, where the diversity increases little over time. From the evidence gathered as part of this body of work, it was believed that community diversity tended to increase with age of the community since the beginning of colonisation.

Three environmentally relevant stressors for communities colonising rocks are desiccation cycles, freeze-thaw cycles and pH extremes. Desiccation and re-wetting cycles are commonly experienced by rock-dwelling microbial communities as a result of rainfall or dew which subsequently dries out. Microorganisms living on rocks often have no shelter from these wetting and drying events as they usually grow exposed on a rock surface, with rock environments sometimes allowing for moisture to be retained for longer, and organisms must be able to withstand this stress in order to survive. Freeze-thaw cycles are prevalent in most temperate or polar environments, and rock-dwelling organisms in these environments again have little shelter to ameliorate the effect of freezing if the temperatures drop below the freezing point. The danger of both desiccation cycles and freeze-thaw cycles is that the cell membranes may be compromised, effectively puncturing the cells, leading to cell death [52]. pH stress works slightly differently, and is an issue for rock-dwelling organisms through elemental leaching from the rock or acid rain altering the geochemical environment beyond the pH in which cells can grow. The rock may provide some buffering, but there is little other matter than can act to shield or buffer the cells from experiencing a sharp change in pH if it occurs. Organisms battle pH stress by maintaining a circumneutral pH inside the cell by either pumping in or out protons or preventing protons from entering the cell. pH stress does not necessarily kill organisms, but may prevent them from growing. If pH stress is so severe that it has a debilitating effect on the cells, this is typically because of damage to molecules, or protein unfolding in the case of low pH [52]. There are additional stresses that are pertinent to rock-dwelling communities, but these three are particularly common and hence chosen for investigation here.

In this chapter, an experiment to study the effect of environmental stress upon two

rock communities of different ages is described. In this study, we use an existing long-term experiment of colonisation of rocky environments (see Chapter 3) to study the effects of environmental stress on microbial communities at different stages of colonisation. Here we compare the survival after environmental stress on communities that have assembled over 2 months ("new" samples) and 14 months ("old" samples), respectively. The experiment tests three environmentally relevant stressors for communities colonising rocks: desiccation cycles, freeze-thaw cycles and pH, over a period of two weeks.

4.2 Unanswered Questions and Hypotheses

In order to further our understanding of how environmental stress affects microbial ecosystems, a few specific unanswered questions were addressed through this experiment.

- Does a microbial community withstand environmental stress better in the early stages of community assembly than later on? A comparison was made between a rock community at two and fourteen months of colonisation. The working hypothesis is that the early community would be better at withstanding environmental stress, as it is composed of pioneer organisms that are often flexible and able to cope with various adverse conditions.

- Does rock type influence viability of a microbial community undergoing environmental stress? Viability after being subjected to environmental stress was compared for two end-member igneous rock types, granite and gabbro. It was hypothesised that although the communities might be different in the two rock types, no net difference in viability would be observed after the communities were subjected to environmental stress.

- In a rock community, which species can survive repeated stressors? Isolated strains were identified after the end of the desiccation stress experiment and the freeze-thaw stress experiment from colonies growing on yeast extract agar plates. The working hypothesis is that the species that grow at the end of the experiment would be hardy, stress-tolerant organsisms that are known to exist in the atmosphere or in soil communities. Some of these species may correspond to those that are prevalent in the community in

the colonisation experiment (Chapter 3) used as the starting point for this study.

4.3 Methodology

For this experiment, samples were taken from an existing long-term study of colonisation of rocky environments, enabling comparisons of the effect of environmental stress on four different microbial communities: 2-month old communities growing in either granite and gabbro, and 14-month old communities growing in either granite and gabbro. These communities came from outdoor microcosms, set up as described in Chapter 3. Very briefly, this experiment consisted of sterile outdoor rock microcosms. The microcosms consisted of 50ml Falcon tubes with a hole in the bottom, to allow for flow-through of rainwater. Each microcosm contained 6.25g of crushed rock of either granite or gabbro, two of the main constituent rock types of the Earths crust. The microcosms were placed in a random grid in a box on top of the James Clerk Maxwell Building at the University of Edinburgh, and were left uncapped in order to be colonised by the atmosphere through wind and rain. Sacrificial samples were collected for analysis every 1-2 months. At every data time point, some new, sterile microcosms were placed on the grid, and collected with the samples from the next time point. This allowed for the monitoring of seasonal variation, such that a continuous record of immigration into the system was kept. For this environmental stress experiment, half the samples had been exposed to the environment for 14 months ("old samples") and the other half for 2 months ("new samples").

The samples were collected from the field on 15 May 2017, and kept at 4°C until 19 May 2017, when the stress experiment was initiated. Nine microcosms were collected of each of the conditions Granite old, Gabbro old, Granite new and Gabbro new. The samples were mixed together in triplicate for other analyses in the original experiment (Chapter 3), and then 2g taken out of each triplicate tube and mixed together for each of the four conditions, such that there was one tube with 6g for each of Granite old, Gabbro old, Granite new and Gabbro new. To each tube was added 18ml (3:1 %w/v) of 0.2% yeast extract (YE) medium (0.2g yeast extract in 1l water), which was mixed by hand for about 10mins and then allowed to settle for 10mins. Yeast extract was used as it is a generalist, varied source of nutrients that supports growth of a wide range of organisms. It essentially consists of dead organisms and thus approximates natural microbial

biomass. This medium is known to successfully culture organisms originating from similar colonisation experiments of artificial, sterile environments [53]. Yeast extract provides a wide range of carbon sources and nutrients for organisms to use, but is also quite a rich nutrient source, and would thus introduce a potential bias in not favouring organisms that require a nutrient-poor environment in which to grow. The community may also be dominated by faster-growing organisms if a nutrient-rich medium is used, as slower-growing organisms may not be able to compete for space in a finite-volume or finite-area system where there are many faster-growing organisms present. All growth media have a potential bias, and in this case a medium which was known to work well within similar studies was chosen. An aliquot of 9ml of the supernatant was taken out into another tube and mixed thoroughly by hand, before being distributed into 1ml aliquots in 1.5ml Eppendorf tubes. Of these, 1 tube was used as control samples, 3 tubes for freeze-thaw tests, 1 tube for pH tests and 1 tube for desiccation tests.

In all three stress experiments (pH, freeze-thaw and desiccation), initial and final number of cells were measured using CFU counts on 0.2% yeast extract agar plates (2% agar). Plates were inoculated using the spread plate technique, and each sample was tested in triplicate.

The desiccation and freeze-thaw samples were treated using the same daily time points. At these time points, the designated samples were subjected to another cycle of either re-wetting or freeze-thaw, respectively. The samples were plated out after 1, 6 and 9 days in order to measure growth. Plates were incubated at ambient room temperature (21°C) for one week before being counted.

4.3.1 Control samples

The control samples were plated using the spread plate technique onto 0.2% YE agar plates at the start of the experiments. Serial dilutions were conducted and plated as follows: x1, x10, x100, x1000, x10000 and x100000 times dilution. CFU counts were measured after 1 week of incubation at 21°C.

4.3.2 pH

In order to test the effects of pH on the growth of the culturable portion of the rock communities, agar plates of different pH were made up. The pH values

tested were 4.5, 5.5, 6.5, 7.5, 8.5, 9, 9.5, 10. Without adjustment, 0.2% YE agar has a pH around 7.1. The pH of the medium was adjusted using either NaOH or HCl before autoclaving and pouring plates. Samples were spread in triplicate onto plates immediately from community working stocks without dilution.

4.3.3 Desiccation

Many microbial communities in rock environments experience periodic rainfall or wetting from dew, followed by periods of drought or desiccation. In certain environments, these cycles are very regular, for instance diurnal cycles of wetting and drying in desert communities, whereas in other environments the cycles are sporadic or irregular [11]. In the local climate of eastern Scotland where these experiments were set up, wetting and drying cycles are frequent, although the drying process can sometimes be retarded due to the high humidity. Thus, an experiment was set up to study the effect of desiccation on viability of a microbial community in the lab, using a desiccation chamber at ambient lab temperatures.

The samples were put in the desiccation chamber at the start of the experiment. The desiccation chamber (Thermo Scientific, Waltham, MA, USA) consists of a large Nalgene polycarbonate container filled with silica gel that absorbs moisture at the bottom, keeping the atmosphere in the chamber dry and ensuring that the samples dry out quickly. Every day the samples were removed from the desiccation chamber and re-wetted with 1ml of 0.2% YE medium, before being placed back into the desiccation chamber. An aliquot was removed and plated after 1, 6 and 9 days. Dilutions were made at $x10^2$, $x10^3$, $x10^4$, $x10^5$ after 1 day, and at $x10^2$, $x10^4$, $x10^6$, $x10^8$, $x10^{10}$ after 6 and 9 days, as substantial growth was observed after 1 day, thus it was necessary to increase the level of dilution in order to be able to count the CFUs. CFUs were counted after one week, but it was only possible to get reliable data from time point 1, as there was too much growth on the plates for subsequent time points.

4.3.4 Freeze-thaw Cycles

In the natural environment, many rock communities would experience seasonal and diurnal changes in temperature. In many areas outside the tropical region, temperatures can frequently drop below freezing. On exposed rock surfaces,

microbial communities would be exposed to repeated freeze-thaw cycles in certain climates. This would be the case for climates such as Scotland, where these experiments were carried out. Hence, an experiment was set up in order to test the survival of the rock communities after repeated freeze-thaw cycles, using a lab freezer at -20°C as the frozen environment and ambient room temperature at 21°C for thawing.

Samples were placed in the freezer and exposed to nine overnight freeze-thaw cycles. Each day the samples were removed from the freezer and left at room temperature for about 30mins to thaw. Each sample was inspected visually to ensure it was fully thawed before being placed back into the freezer. Samples were plated out after 1, 6 and 9 days on yeast extract plates to dilutions of x10, x100, x1000, x10000 and x100000, and CFUs counted after 1 week.

4.3.5 Colony PCR and sequencing of selected isolates

In order to identify the composition of the community that was viable after being subjected to the various stressors, individual isolates were analysed through colony PCR and Sanger sequencing. Firstly, colonies were selected from the output agar plates. The widest possible range of colours and morphologies were picked in order to get a comprehensive and representative sample. These isolates were then streaked out on fresh YE agar plates using an inoculation loop in three iterations in order to ensure pure colonies. After the third transfer, colony PCR was performed.

PCR reactions were conducted using the GoTaq G2 Colourless Master Mix and reagents listed in Table 4.1, and primers 27F and 1389R as seen in Table 4.2. Cells were transferred carefully from the colony on the plate to a 0.2ml PCR tube containing 25μl volume of reagent using a sterile pipette tip. PCR runs were performed on a thermocycler (G-Storm GS1, Gene Technologies Ltd., Braintree, UK) according to the protocol outlined in Table 4.3. The PCR products were run on a 1.2% agarose gel stained with SYBR Gold DNA binding dye (Life Technologies, UK) to confirm the successful amplification of DNA in each sample before downstream analysis. After PCR, the DNA was purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, following the protocol for use with a microcentrifuge (Thermo IEC Micro CL 17 centrifuge, Thermo Fisher Scientific, UK). For the final elution, 30μl of buffer EB was used.

Table 4.1 *Reagents used for in-house PCR*

Reagent	Amount
Forward primer (0.01mM stock)	1.0μl
Reverse primer (0.01mM stock)	1.0μl
Master Mix	12.5μl
MQ Water	10.5μl
Total volume	25μl

Table 4.2 *Primers used for checking DNA extraction yield*

Type	Gene	Forward name	Forward sequence	Reverse name	Reverse sequence
Bacterial	16S	27F	AGAGTTTGATCMTGGCTCAG	1389R	ACGGGCGGTGTGTACAAG

DNA concentrations in each sample were measured using NanoDrop^{TM} (Thermo Fisher Scientific, UK). The samples were prepared for sequencing by mixing an aliquot of each sample with water and 0.33μl of either primer 27F or 1389R at 0.01mM stock to a total DNA amount of 40ng per sample. Each sample was submitted for sequencing in duplicate, one for the forward and one for the reverse primer. Sanger sequencing was conducted by Edinburgh Genomics (Edinburgh, UK), who performed BigDye reactions, clean-up and sequencing on an ABI 3730XL capillary sequencing instrument.

4.3.5.1 Data analysis of sequenced isolates

Data from Sanger sequencing was downloaded as chromats in .ab1 format. Forward and reverse strands were assembled using DNA Baser (Heracle Biosoft, Romania) [210], and the resulting contig identified to the closest match using BLAST [4].

Table 4.3 *PCR program for in-house PCR*

Step	Temperature	Duration	Number of repeats
Initial denaturation	94°C	4 min	
Denaturation	94°C	0.5 min	35 times
Annealing	54°C	0.5 min	
Elongation	72°C	0.5 min	
Final elongation	72°C	5 min	
Cooling	4°C	10 min then hold	

4.4 Results

Data for this experiment consist of viability changes over time as measured by CFU counts and identified isolates from the various time points.

4.4.1 Control samples

Growth was measured at the start of the experiment for control samples that were not subjected to any stress. CFU counts were found to be on the order of 10^7 CFUs/ml for all samples, with the counts in the older gabbro samples being about five times higher than the other three sample types (Figure 4.1).

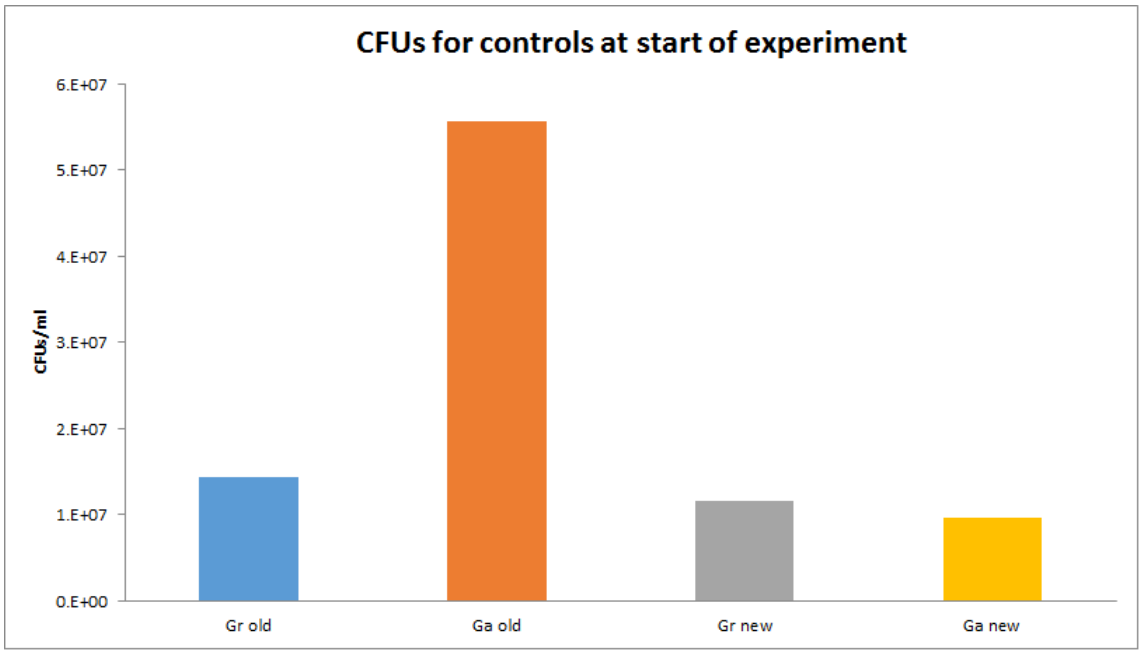


Figure 4.1 *Growth of controls at start of experiment, as measured by CFUs/ml. Gr=granite, Ga=gabbro, old=14 months, new=2 months.*

4.4.2 pH

Growth was observed for both rock types and for both old (14 months) and new (2 months) samples at all pH values tested, namely 4.5, 5.5, 6.5, 7.5, 8.5, 9, 9.5, 10. It was not possible to further investigate how many isolates of each community grew under each condition as all plates became overgrown, but where growth was recorded there was at least one isolate that could grow. Growth at

x1 (no dilution) was too high to be quantified using CFU counts. As these were overgrown with colonies no attempt was made to count the number of CFUs, but this was expected as no dilution series was made, as the goal was to identify the ability of the community to grow in the selected pH range. The results show that at least one isolate of all samples can grow well in the pH range 4.5-10.

4.4.3 Desiccation

For the desiccation experiment, instead of an expected decrease in viability after repeated desiccation cycles, viability was observed to increase for all communities from the four different conditions. In Figure 4.2, it can be observed that the viability has increased the most in the new (2 month) granite samples, followed by the new (2 months) gabbro samples, with a smaller amount of growth in the old (14 months) samples for both rock types. These increases between the start of the experiment and the first time point were all statistically significant at the 0.05 confidence level, for new 2-month granite samples (t-test, p=0.0000173), new 2-month gabbro samples (t-test, p=0.000397), old 14-month granite samples (t-test, p=0.00236) and old 14-month gabbro samples (t-test, p=0.00255). It was only possible to measure growth after day 1, as too much growth had occurred at subsequent time points, swamping the agar plates such that counting was impossible, even though serial dilutions down to x10^{10} were conducted.

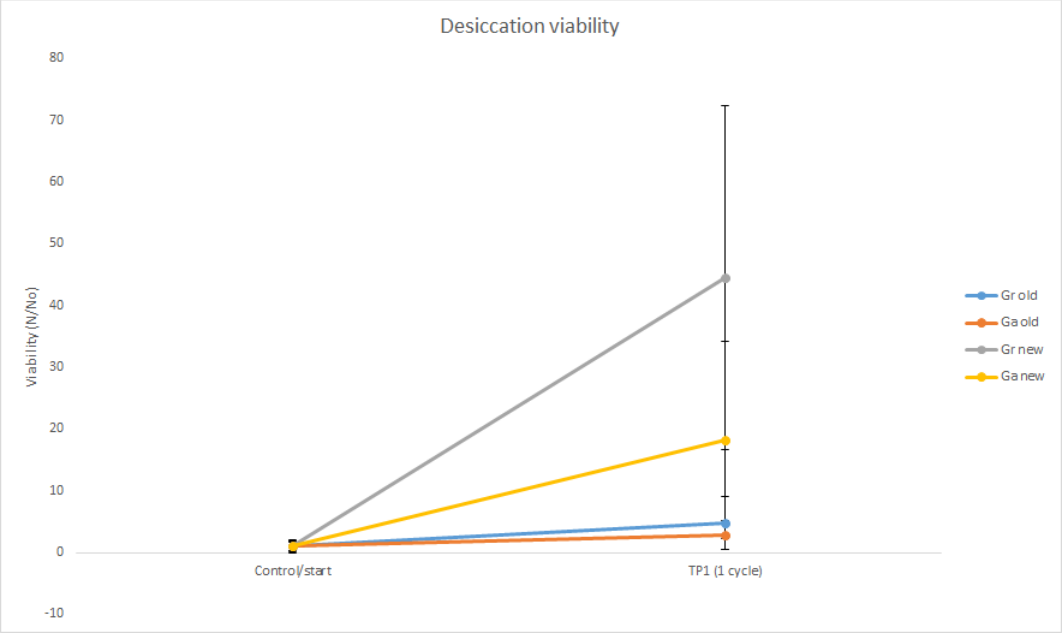


Figure 4.2 *Viability after 1 day of desiccation.*

Regarding the colours seen in the colonies on the plates, some distinct differences are observed between samples. In general, some coloured colonies are seen after 1 cycle, whereas after 6 and 9 cycles only white or transparent colonies are observed. After 1 day, yellow colonies are seen in the new granite and gabbro samples and orange colonies in the old gabbro samples, whereas the old granite samples only contained white or transparent colonies. In the old gabbro samples, the orange colonies seem to only be growing inside other white colonies.

Some fungal growth is observed on the plates when incubated at room temperature for 1 week, however, this growth is significantly less than in the freeze-thaw experiment, as discussed in Section 4.4.4.

The results from the desiccation experiment indicate an unforeseen flaw in the methodology, leading to unreliable data and hence an inability to accurately test for the hypothesis in question. An increase in viability was seen with each re-wetting and desiccation cycle, indicating that growth was taking place (see Figure 4.2). The likely reason for this is that the cells had a chance to grow in the liquid YE medium before they were completely desiccated. Thus, the time for desiccation to occur fully was much less than the doubling time for the community, or at least for some specific species that thrived in the presence of YE liquid medium. The pellets were completely desiccated each day before more medium was added, but the time it took for the medium to dry out is unknown.

4.4.3.1 Isolate Identification

From the colonies growing on the YE plates after 1 desiccation cycle, 13 isolates were analysed and identified using colony PCR and Sanger sequencing, as seen in Figure 4.3. Of these, four isolates were identified as *Sphingobacterium sp.*, three as *Pseudomonas sp.* and one of each of *Stenotrophomonas sp.*, *Massilia aurea*, *Chryseobacterium sp.*, *Sanguibacter inulinus*, *Microbacterium testaceum* and *Exiguobacterium sp.* The colonies that were assumed to be duplicates (where one colony was transferred from another in the purification process) were identified as the same species, or as the same genus if no species identification could be made.

	Experiment	Colour	Duplicate colony	Rock type	BLAST match	Notes
H16	Desiccation	Clear		Granite	Stenotrophomonas sp.	
H17	Desiccation	White		Gabbro	Sphingobacterium sp.	
H18	Desiccation	Clear		Gabbro	Pseudomonas sp.	
H19	Desiccation	Clear		Gabbro	Pseudomonas sp.	
H20	Desiccation	Clear		Gabbro	Pseudomonas sp.	
H21	Desiccation	Yellow	Same as H28	Granite	Sphingobacterium sp.	
H22	Desiccation	Yellow		Granite	Massilia aurea	First nine matches were uncultured
H23	Desiccation	Yellow		Granite	Chryseobacterium sp.	First match was uncultured
H24	Desiccation	Yellow		Granite	Sanguibacter inulinus	First match was uncultured
H25	Desiccation	Clear		Granite	Sphingobacterium sp.	
H26	Desiccation	Yellow		Gabbro	Microbacterium testaceum	Identified from R strand only
H27	Desiccation	Orange		Gabbro	Exiguobacterium sp.	
H28	Desiccation	Yellow	Same as H21	Granite	Sphingobacterium sp.	Identified from F strand only

Figure 4.3 *Identified CFUs growing after 1 day of desiccation.*

4.4.4 Freeze-thaw Cycles

In the freeze-thaw experiment, a decrease in viability was observed for all samples after the final time point of nine freeze-thaw cycles, as seen in Figure 4.4. The biggest decrease is observed in the viability for the old samples for both rock types, with the highest viability retained by the new granite samples, followed by the new gabbro samples. Notably, this is is the same pattern of growth viability as observed in the desiccation experiment. The results from the freeze-thaw experiment suggest that the newer samples are more resistant to stress than the old samples, and that the new granite samples have the highest retention of viability under freeze-thaw. The new granite samples also retain the highest viability for desiccation cycles, where the new granite samples experience the highest levels of growth. These results must be treated with some caution as the error bars (standard error) are large. The decreases in viability are, however, statistically significant at the 0.05 confidence level for new 2-month gabbro samples (t-test, p=0.00209), old 14-month granite samples (t-test, p=0.000463) and old 14-month gabbro samples (t-test, p=0.0000304), but not for the new 2-month granite samples, where the viability is around 90%.

From a qualitative analysis, mainly coloured colonies (yellow, orange and pink) were observed on plates from 6 or 9 cycles of freeze-thaw, whereas mainly white and yellow colonies were visibly growing on the plates after 1 cycle.

Fungi are observed to grow on some plates after a week of incubation. Notably, the old granite samples have the most fungi, followed by some on the old gabbro samples, and hardly any fungal growth is observed on the new granite and gabbro samples. It is also noted that there is significantly more fungal growth on the agar plates in the freeze-thaw experiment than in the desiccation experiment.

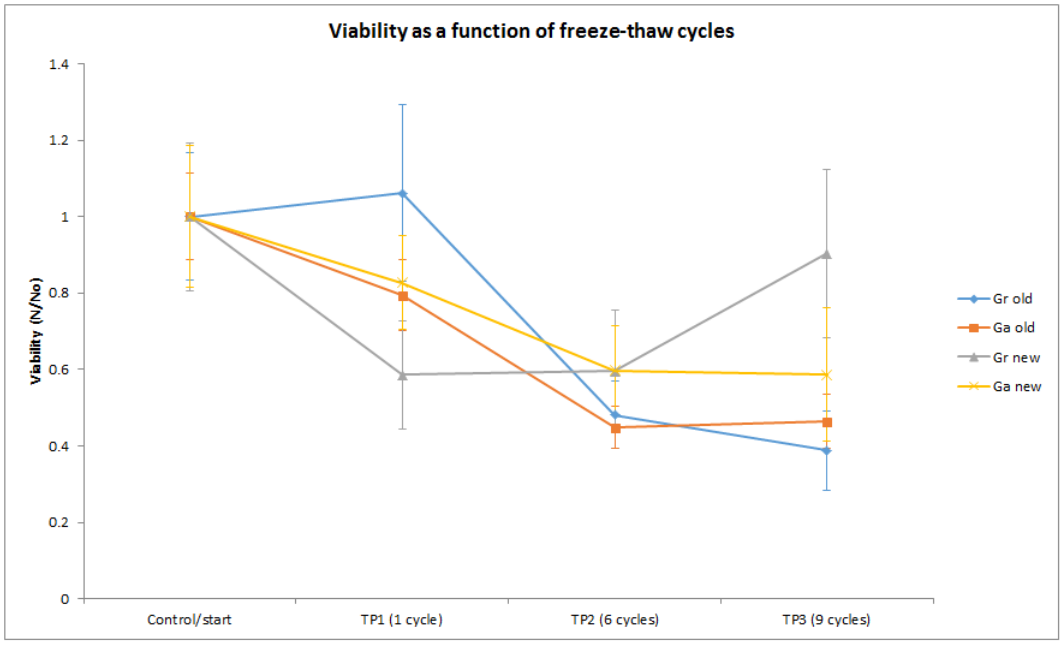


Figure 4.4 *Viability at each time point after repeated freeze-thaw cycles.*

4.4.4.1 Isolate Identification

From the colony PCR, identification was possible of 13 isolates from a total of 15 colonies. Two colonies failed the sequencing step for both the forward and reverse strand, meaning that identification was impossible. Of the isolates that were possible to identify using BLAST, three match up with *Sphingomonas faeni* and another four as *Sphingomonas* sp., two as *Clavibacter* sp., two as *Arthrobacter agilis*, and one each of *Microbacteriaceae* sp. and *Chryseobacterium* sp.

	Experiment	Colour	Duplicate colony	Rock type	BLAST match	Notes
H1	Freeze-thaw	Light pink	Same as H14	Granite	Clavibacter sp.	
H2	Freeze-thaw	Yellow		Gabbro	Microbacteriaceae sp.	
H3	Freeze-thaw	Pink		Gabbro	Arthrobacter agilis	
H4	Freeze-thaw	Light pink	Same as H4	Gabbro	Sequencing failure	Sequencing failed for both strands
H5	Freeze-thaw	Light pink	Same as H5	Gabbro	Sequencing failure	Sequencing failed for both strands
H6	Freeze-thaw	Pink/red		Gabbro	Arthrobacter agilis	
H7	Freeze-thaw	Yellow		Gabbro	Chryseobacterium sp.	
H8	Freeze-thaw	Orange	Same as H9	Gabbro	Sphingomonas sp.	Identified from F strand only
H9	Freeze-thaw	Orange	Same as H8	Gabbro	Sphingomonas sp.	First match was uncultured
H10	Freeze-thaw	Orange		Granite	Sphingomonas faeni	Identified from F strand only
H11	Freeze-thaw	Yellow		Granite	Sphingomonas sp.	
H12	Freeze-thaw	Orange		Granite	Sphingomonas sp.	
H13	Freeze-thaw	Yellow		Granite	Sphingomonas faeni	First two matches were uncultured
H14	Freeze-thaw	Light pink	Same as H1	Granite	Clavibacter sp.	Identified from R strand only
H15	Freeze-thaw	Yellow		Granite	Sphingomonas faeni	

Figure 4.5 *Identified CFUs growing after 9 freeze-thaw cycles.*

4.4.5 Fungal growth

Some fungal growth is observed in all samples on the agar plates. In general, more fungal growth was observed on the freeze-thaw plates than on the desiccation plates. In the freeze-thaw experiment, most fungal growth is seen in the old granite samples, followed by a little less in the old gabbro samples, with very little growth visible in the newer samples.

Fungal growth on agar plates may confound the quantification of bacteria present in the samples. Notably, fungi growing on plates may obscure or inhibit the growth of bacteria, making estimates of viability of a sample more difficult. Ways to avoid fungal growth from a community sample could involve growing the plates at a lower temperature, or incubating the plates for a shorter time, however, these methods may also inhibit the bacterial growth under study. In general, fungal growth on agar plates is difficult to avoid completely with these types of environmental rock-dwelling samples. In this instance, no further analysis was conducted on the fungi present.

4.4.6 CFU colours

It is observed that the freeze-thaw samples had a much wider variety of different CFUs growing on the YE plates. The CFUs visible on the freeze-thaw plates were of several different colours - yellow, orange, white, red and pink. The CFUs on the desiccation plates were mainly white or transparent. In addition, many of the colonies on the desiccation plates appeared to be the same species, as they grew in distinctly transparent doughnut-shaped colonies with raised edges and a depression in the middle.

4.4.7 Results summary

It is found that after 9 freeze-thaw cycles, there is a loss of viability in both the old and new communities from both rock types. The communities are able to grow on yeast extract agar of all pH tested, from pH 4.5 to pH 10. Desiccation shows an increase in viability after one cycle, likely due to growth before desiccation was completed. Heterogeneity in sampling might also somewhat influence the results, as it is possible that the samples may have been imperfectly mixed before aliquots

were removed. Every effort was taken to reduce this sampling heterogeneity, or potential of each aliquot containing a slightly different community due to imperfect mixing, but due to the nature of the samples involving rocks, a perfectly homogeneous sample cannot be guaranteed. This means that if an aliquot is removed from the sample, a slightly different bacterial diversity may be present and subsequently cultured or analysed in the downstream processes. If this sampling heterogeneity is a real effect in a system, it may mean that the difference is increased between communities within triplicate samples or across conditions, and also that the overall diversity present may be overestimated.

4.5 Discussion

This experiment was set up to study the differences in viability in response to stress at two different stages of community assembly. Identical microcosms that had undergone colonisation for two and fourteen months respectively were tested under the same environmental stress conditions. The use of solid culturing techniques enabled comparisons of viability in response to stress to be made.

4.5.1 Does a microbial community withstand environmental stress better in the early or late stages of community assembly?

In the freeze-thaw experiment, a higher viability is seen in the 2-month granite and gabbro samples compared to the 14-month samples after being subjected to stress (Figure 4.4). This indicates that the 2-month samples are better able to cope with environmental stress. This is in line with the hypothesis that the younger communities would be better at surviving stressors, as the community would be made up mostly of pioneer species that are versatile and able to adapt to different environmental conditions.

These results are different from some other reports in the literature, where a young community is thought to be more susceptible to drastic environmental changes or stress [218], [14]. These theories pertain to simple communities consisting of only a few species, or only a few species for each function or trophic level, which could mean that if stress creates population crashes or

drastic reduction in certain species, the whole community may collapse. In this study it is known, from the results in Chapter 3, that the 2-month communities are in fact already complex communities that consist of a multitude of species. The 2-month communities are dominated by *Janthinobacterium* and an unidentified genus of the Oxalobacteraceae family, but otherwise consist of a complex community of many different species. The 14-month communities are dominated by Cyanobacteria in the form of *Leptolyngbya*, but again form a complex community overall. There are no great differences in the Shannon and Simpson indices between the communities. This may explain why drastic effects in response to stress are not observed for the 2-month communities. In order to capture the behaviour of a simple, young community one may need to study a much younger community, of ages on the order of days to weeks.

These results must be treated with some caution, especially in the case of the desiccation experiment, where for all samples viability was higher after being subjected to stress than before (Figure 4.2). This is likely caused by the fact that the addition of nutrients in the liquid yeast extract used to re-wet the samples was enough to enable the community, or at the very least one or a few species, to grow rapidly and thus increase in viability before desiccation could take place.

4.5.2 Does rock type influence viability of a microbial community undergoing environmental stress?

The results from both the desiccation stress and the freeze-thaw stress experiments show a higher viability for granite than gabbro for the new samples, while for the older samples this difference is not obvious (Figures 4.2 and 4.4). This suggests that the community from the granite substrate is better at withstanding environmental perturbation or stress in the early stages of community assembly. It is possible that granite provides a harsher environment with less nutrients freely available, hence these communities might be more adaptable and resistant to changes [190].

4.5.3 In a rock community, which species can survive repeated stressors?

The isolates surviving after being subjected to environmental stress in this study are different for the desiccation experiment and freeze-thaw experiment, but there is also some overlap as some genera are recorded to be viable under both conditions. Here, only the freeze-thaw experiment exerts a detrimental stress on the community, with a resulting decline in overall community viability.

4.5.3.1 Viable genera and species in the desiccation experiment

For the desiccation experiment, the isolates are identified as follows: four isolates as *Sphingobacterium sp.*, three as *Pseudomonas sp.* and one of each as *Stenotrophomonas sp.*, *Massilia aurea*, *Chryseobacterium sp.*, *Sanguibacter inulinus*, *Microbacterium testaceum* and *Exiguobacterium sp* (Figure 4.3).

Sphingobacteria belong to the phylum Bacteroidetes and are characterised by high concentrations of sphingophospholipids. They are Gram-negative and nonsporeforming chemolithotrophs that often form yellow colonies after some days growing on agar plates. In this study, *Sphingobacterium* colonies were observed to be both yellow and clear after some days, indicating that they were likely to be at least two different species [237]. The fact that they are chemoorganotrophic means that they require organic matter for growth, thus, in order to live in a rock environment they need to obtain the necessary nutrients from organics in the environment. The pigment suggests that they have some UV tolerance necessary for transportation through the atmosphere and living on exposed surfaces.

Pseudomonas is the second most common genus of the identified isolates, and belong to the class Gammaproteobacteria. They are rod-shaped, Gram-negative, flagellated, aerobic, non-spore forming, catalase-positive and oxidase-positive and are found in a variety of different environments such as soils but also as plant and animal pathogens [226]. Some species are known to be ice-forming and hence perform an important function in the atmosphere in the nucleation of ice and snow [47]. This is consistent with the finding that these microbes are viable after experiencing environmental stress, although none are actually identified among the survivors of the freeze-thaw cycles.

Stenotrophomonas are also Gram-negative Gammaproteobacteria. They are

common in many environments, including soils and can be found to be promoting growth or living in symbiosis with plants. Some species are opportunistic human pathogens and common hospital bacteria responsible for outbreaks [120].

Massilia aurea is a Gram-negative species of Betaproteobacteria, which are rod-shaped and nonsporeforming aerobes. The colonies have a yellow colour, and the species was first isolated in drinking water from Sevilla, Spain, although other members of the genus have been isolated from soil samples [97].

Chryseobacterium sp. are found to be viable in both the desiccation and the freeze-thaw experiment. They are part of the Bacteroidetes and hence Gram-negative and are mostly found in soils but occasionally as opportunistic human pathogens [2].

Sanguibacter inulinus belong to the Actinobacteria and are Gram-positive, irregularly rod-shaped, motile organisms. They form yellow colonies on tryptose soy agar plates and are facultative anaerobes that were first isolated from the blood of apparently healthy cows [196].

Microbacterium testaceum is another member of the Actinobacteria, and is typically found as an endophyte associated with plants, where it does not cause disease [184]. It is Gram positive and has been isolated from many important agricultural crops such as potato, corn and sorghum [30].

Exiguobacteria is a versatile extremotolerant, Gram-positive, nonsporeforming genus belonging to the Bacillus class of the Firmicute phylum that has been isolated from both cold environments such as permafrost and glaciers as well as Yellowstone hot springs [257], [58]. They have been isolated from many environments around the world, including lakes, soils, biofilms, stromatolites and hydrothermal vents. Specific species within the genus are also known to be halotolerant, UV tolerant, high and low-pH tolerant and heavy metal tolerant, leading to suggestions of being of agricultural and industrial importance [142].

All the isolated genera and species from this experiment are environmental taxa with some tolerance for extreme conditions. This is consistent with the environment they were isolated from, and the environmental perturbation they have been subjected to. No isolates appear to have previously been identified as having specific desiccation tolerance, but it is not surprising that environmental organisms can withstand and even grow after one desiccation and re-wetting cycle as they likely experience desiccation in the natural system.

4.5.3.2 Viable genera and species in the freeze-thaw experiment

In the freeze-thaw experiment, the identified viable isolates are two colonies of *Clavibacter sp.*, four colonies of *Sphingomonas sp.*, three colonies of *Sphingomonas faeni* and one colony each of *Microbacteriaceae sp.*, *Chryseobacterium sp.* and *Arthrobacter agilis* (Figure 4.5).

The genus *Clavibacter* belongs to the Actinobacteria and currently contains only one known species, *Clavibacter michiganensis*, and five subspecies. This species is a Gram-positive plant pathogen of economic interest as it can cause major damage to tomato and potato production. Transfer is thought to occur via contaminated seeds, stakes, tools or soils [236]. In this case of this experiment, a contaminated seed entering the experimental system is the only plausible transfer mechanism, alternatively, the organism could have been dispersed freely in the atmosphere. There is some evidence to suggest that species in this genus may be unable to persist in the absence of plant material, which the strain would have done here [22]. It is possible that the strain existed endophytically in the soil community before the start of this experiment, but it was found to grow on agar plates in the absence of plants after being subjected to the freeze-thaw treatment.

Sphingomonas is a genus of common soil bacteria that are Gram-negative, rod-shaped, aerobic, mostly chemolithotrophic (although some can use organics) members of the Alphaproteobacteria. They have been isolated from a wide variety of environments, including rivers, sediments and the rhizosphere and surfaces of plants as well as hospital equipment and corroding copper pipes [266]. They form yellow colonies on agar plates, which is consistent with the observations in this experiment.

Sphingomonas faeni are orange-pigmented psychrotolerant bacteria that are easily transported by dust particles. There is some evidence to suggest that *Sphingomonas faeni* may have a role to play in preventing food spoilage at low temperatures [170], [34].

The *Microbacteriaceae* family belongs to the Actinobacteria and are Gram-positive soil bacteria and sometime plant pathogens [20]. Notably, the genera *Clavibacter* and *Microbacterium* identified in this study belongs to this family.

The species *Arthrobacter agilis* is a psychrotrophic soil organism in the Actinobacteria phylum. It is Gram-positive, motile and nonsporeforming, forming matte colonies on agar plates, and can grow in water, soil and on human skin

[154]. The species has plant growth-promoting and anti-fungal properties [254], [253]. Research on strains from Antarctic sea ice showed increased carotenoid production with decreased temperature, and also observed the species to have some resistance to salt stress [91].

A colony of a *Chryseobacterium sp.* was found also in the desiccation experiment and is discussed in more detail in Section 4.5.3.1.

Thus, several of the isolates from the freeze-thaw experiments are identified as species with a known tolerance of freezing or other extreme conditions. Many environmental organisms in temperate climates frequently experience freeze-thaw cycles, and must have some way of coping with these in order to survive. The weather data from the colonisation experiment from which these organisms originate (Chapter 3) show that the samples experience frequent freeze-thaw cycles in natural conditions, and that freezing occurred on 48 days during the 18-month experiment. These observations are consistent with the fact that the communities in this freeze-thaw experiment experienced some decrease in viability, but did not experience a total population collapse.

4.5.4 Fungal growth

Some fungal growth is observed in all samples on the agar plates, with more growth observed in the freeze-thaw than in the desiccation condition, suggesting that either fungi proliferates under repeated freezing and thawing, or that they are out-competed by the growth of bacteria in the desiccation experiment.

In the freeze-thaw experiment, fungal growth is observed to be more extensive in the older samples than the newer samples, most likely as they formed a larger part of the community at the outset of the stress experiment due to the age of the microcosms, however, no significant fungal growth was observed in the control samples. There is no known *a priori* reason why there should be more fungal growth in the granite than gabbro samples, as is observed to be the case.

4.5.5 Visible pigmented colonies and atmospheric input

Some interesting observations can be made from this experiment regarding the colours of the colonies growing on the agar plates. Firstly, many pigmented

colonies are observed, which is consistent with these being atmospheric or rock-dwelling taxa, which typically have pigments to shield against UV radiation or reactive oxygen species while exposed during atmospheric transport or on rock surfaces [215]. It is also possible that in some species the pigment production is associated with psychrotolerance as they help stabilise the membrane, as for instance in *Arthrobacter agilis* [91].

Another interesting finding is that pigments only appear to be expressed when the agar plates are not overcrowded. At low dilutions where the plates are so overgrown that CFU counts are impossible colonies are observed to be either transparent, white or buff coloured. At higher dilutions, where CFUs are well defined and distinct and in numbers low enough to count, many, if not all, colonies appear pigmented in yellow, red, pink and orange. This phenomenon is also observed in the related experiments as described in Chapters 3 and 6. These findings may require more investigation, as this phenomenon is not well studied in the existing literature.

4.5.6 pH tolerance of rock communities

In this experiment, at least some members of all sample communities are able to grow under all pH values tested, from 4.5-10.0. There is no evidence of a decrease in growth towards the ends of the range, but as these systems are complex communities it is possible that even one or two taxa that are able to grow at the designated pH can be enough to generate substantial growth even at a high or low pH. In other words, it may be the case that different isolates can grow at different pH ranges, thus the community as a whole cover the range being tested. As this is true for both ages of the community, these data suggest that a large pH range tolerance is possible even in the early stages of community assembly. Rock communities like these are often well adapted to a larger range of environmental conditions, which is corroborated by these findings.

4.6 Limitations and future directions

There are several valuable lessons from this study regarding factors that can be improved if this type of experiment is repeated in the future.

4.6.1 Liquid medium for re-wetting samples for the desiccation experiment

The most important factor to consider if repeating the desiccation experiment would be to use a different medium to re-wet the dried pellets with after each cycle of desiccation. In this case, it appears that the use of yeast extract medium was too rich for the community, such that some species were able to grow while desiccation was taking place, leading to an increase in viability after each desiccation cycle. Here, it was decided to use yeast extract as the samples were plated out on yeast extract agar plates, which was consistent with the experimental procedures from which these samples originated, where cell counts were conducted on yeast extract agar plates(see Chapter 3). Other options would be to use distilled water, a buffered solution such as PBS, or a minimal medium. Distilled water was not used here as it has the potential of killing cells by disrupting the membranes if there are not enough solutes in the solution. Using a solution such as PBS which contains some salts would be one way around this problem. It is possible that in this case distilled water would have been possible to use, as some solutes would have remained with the cells in the pellet after the original clean-up steps. Using only distilled water or a minimal salts solution without a nutrient source could however also result in a loss of viability due to starvation in the absence of nutrients rather than as a result of the detrimental effects of desiccation, thus confounding the results. Using a minimal medium such as M9 is another option, but even these may have enough nutrients for the community to grow in the time it takes for desiccation to occur.

4.6.2 Testing pH tolerance

It is possible to devise a way of testing a larger range of pH tolerance for further studies of these types of communities. Here, the range was chosen to be that which allowed solid agar plates to be made, as outside this range the agar will not settle, and thus was the largest range which could be tested using the methodology that this experiment was based on. If a larger pH range is desired, other methods such as liquid culturing must be employed.

4.7 Conclusions

In conclusion, this experiment has tested the response of rock-dwelling microbial communities to environmental stress at different stages of community assembly. The results indicate that the communities are better at withstanding environmental stress early in the community assembly, consistent with the hypothesis that the early communities are more versatile and hence able to withstand perturbation. Some evidence appears to suggest that the granite communities are better at withstanding stress than those in gabbro rock. The isolates found to survive under stress are typically soil organisms, many of which are known to be extremotolerant. Thus, the findings from this experiment shed light on the environmental stress response of rock-dwelling microbial communities.

Chapter 5

The importance of priority effects in the assembly of complex microbial communities

Most microorganisms on Earth exist in complex communities comprised of many different taxa. These communities enable efficient cycling of nutrients in complex ecosystems. There are several theories on how these communities are formed, and what factors are important in the process of colonising a new habitat. One unanswered question is how important priority might be; in other words whether organisms that arrive early to a new habitat have a higher chance of survival and success than those that arrive later.

Previous studies have shown that priority effects do play a role in community assembly, although the exact mechanisms and relative importance relating to other factors influencing assembly remain unclear. For instance, in a study involving nectar-colonising yeasts, with pairwise comparisons between different species, the earlier arrival was found to have a negative impact on the growth of the secondary arrival, and it was found that environmental harshness had an effect on the strength of these interactions [251]. Priority effects have also been shown to influence the diversification of an ancestral species into its various morph types [95]. In a study of the colonisation by seeding with a diverse community and immigration from natural sources on artificial pond mesocosms, priority effects were found to be important for the resultant community, alongside stochastic effects [45]. In a natural setting, priority effects work in synergy with other

factors in order to shape the resultant microbial community, and the influence of priority on environmental isolates remains unclear.

An experiment was set up to test the importance of priority effects in a laboratory setting using environmental isolates, in which different strains of coloured isolates were mixed together at different intervals, such that one strain was given some time to acclimatise to the new habitat before the second strain was added. It was hypothesised that the earlier arrival would give an advantage for growth resulting in greater cell number at the time of analysis, as the first isolate has possibly altered the environment for later arrivals. Of three experiments conducted, this was found to be the case in two instances, i.e. priority effects led to an advantage in growth for the earlier arrival, and the later arrival did not achieve the same amount of growth as it did in monoculture over the same timescales. In the last experiment, conducted with different isolates, priority effects were not observed, as one isolate always dominated, regardless of whether it was added first or second. These effects can be explained by a difference in growth rate, where priority effects matter if the growth rates between the two strains are similar, but if the growth rates are vastly different, the faster-growing strain will always out-compete the other if the timescales are long enough.

The experiment was cut short, meaning that more strains were not tested, nor more experiments conducted with the above isolates, as problems with clumping of individual isolates were encountered. Clumping was observed with a many of the isolates that were trialled, and is a well-known phenomenon in environmental organisms to produce biofilms or EPS (extracellular polymeric substances), especially under stress [26], [135], [98], [232]. All strains had been isolated from an existing experiment studying the colonisation of igneous rock. Clumping could potentially have been avoided by using model organisms, however, this was decided against due to a desire to use strains derived from an existing experiment that were known to have a contextual importance. The outcomes of this study illustrate that there can be somewhat of a discrepancy between research conducted on model organisms and the behaviour of environmental organisms, as many environmental isolates may display properties making them difficult to work with using standard laboratory procedures [194], [8], [130], suggesting that more research needs to be conducted on priority effects on organisms relevant for the problem and variables under study.

5.1 Introduction

This chapter chronicles an experiment looking at priority effects in colonisation. The aim was to use artificial communities to answer the question of whether an earlier arrival gives an advantage over later colonists. The strains used for this experiment were isolated from an existing experiment looking at colonisation of rocks (see Chapter 3). The chosen strains exhibited different colours when forming colonies on agar plates, hence enabling differentiation and enumeration of different strains grown in co-culture. The experiment yielded no conclusive results due to different growth rates and clumping of cells in liquid culture, making counts unreliable.

In this experiment, the aim was to study the early stages of community assembly, utilising an artificial community of coloured isolates, isolated from the atmosphere by sampling a well-constrained, initially sterilised, rock environment. The primary question addressed was how community development is affected by the order of colonisation of a sterile rock habitat. By using coloured isolates, one can study community composition by quantifying and comparing growth on agar plates, using the different colours to distinguish between the growth of the different strains. As a further step, this lends itself to studying priority effects in synergy with other influences from the environment, which can be done for instance by using different rock substrates and thus geochemical environments.

Different species of microorganisms have various functions and tolerances which allow them to become successful in different environments and communities. Specifically, environmental variables such as pH, Fe concentration and organics concentration can act as selectors for the emerging community, by allowing certain species to be successful, at the expense of species that are less well adapted to the present environmental conditions [211], [64], [223], [72], [48]. Early colonisers will also often alter the environment for later arrivals as a byproduct of their metabolism [272], [251]. By quantifying the pH, Fe and organics dependence of the different isolates, we can make predictions of how community assembly might differ depending on the order of colonisation and initial conditions.

There are many key parameters that affect microbial growth, either by limiting growth or providing optimal conditions for growth. Some of the limits to life are set by pH, temperature, pressure, salinity, radiation, metal concentrations and organics concentrations [52]. For this study, the focus is on the limits of

pH, Fe concentration and organic carbon concentration. Microorganisms have various tolerances and ranges of pH within which they can achieve maximum growth, and usually an outer range of pH within which they can grow at a slower rate. Organisms with optimal growth below pH 3 are known as acidophiles and those that achieve optimum growth above pH 9 are known as alkaliphiles, while most other organisms have optimum growth conditions somewhere in between these extremes [52]. Microbial growth of heterotrophic organsims is limited or affected by the amount of available organic carbon to use for energy [52], [151]. Different organisms have different nutritional requirements, with some organisms being adapted to nutrient-rich environments (copiotrophs), whereas others are adapted to nutrient-poor environments, and do not grow well if inundated with nutrients [153]. Lastly, Fe can be a limiting nutrient for microorganisms, as it is a bioessential element that is used for most cell functions [64], [223], [116]. Taken together, these factors can be used to better understand the growth dynamics of microorganisms under different environmental conditions.

Previous studies have approached various aspects of community assembly through either computational models, experimental model systems, or a combination of the two. For instance, Kettle et al. [149] (2014) studied the microbial dynamics of the human gut microbiobial communities using a computational model based on bacterial functional groups, and the particular substrate preferences, metabolites and pH ranges of each strain, and found that this model well reproduces the results of experimental investigations. In another study, Pagaling et al. [191] (2014) looked at community assembly in Winogradsky columns, which are a useful tool used in order to culture a large diversity of microorganisms in a contained system, and found that community history can affect the future predictability of the ecosystem. Williams and Lenton [272] (2007) and Williams and Lenton [273] (2008) looked at microbial communities using a computational model system based on a series of interconnected flasks, where the ecosystem tends toward a regime whereby nutrients are efficiently recycled within the system, leading to an increase in the population size, but ecosystems are vulnerable to organisms that alter the environment away from the state to which the majority of the community is adapted. They go on to show that communities which improve their environment are generally more stable, grow to larger sizes, and are better at colonising new space, whereas communities that degrade the environments they are in tend to get smaller and are more susceptible to invasion by other organisms. Lastly, Vannette and Fukami [251] (2014) studied priority effects using yeasts with well-known properties through extensive laboratory studies,

and found that the earlier arrivals into a pristine system negatively affected the growth of a later arrival, and found that if the order of colonisation was reversed, the primary colonist always had advantage. These studies illustrate some ways in which microbial population dynamics can be probed through model systems.

The rationale for this study was to take a dual approach, whereby the system would be probed from both a laboratory experiment and a computer modelling point of view. The goal was to attain environmental isolates and establish their response or requirements for the key parameters of pH, Fe concentration and organics concentration, to perform laboratory mixing experiments with them, and also model the system using the experimental data. In this way, it would be possible to see whether the laboratory results could be accurately modelled, and draw conclusions about the underlying factors affecting the experimental evidence.

5.2 Unanswered Questions and Hypotheses

- Are priority effects strong enough to have a significant effect on the survival of two different species mixed together at different times? In other words, in microbial community assembly, does it matter who gets there first? This can be tested using two isolate strains, mixed together in the same medium at different times with appropriate controls, and measuring growth of both strains after a set period of time. Conducting an experiment with strain A given priority over strain B, and another experiment with strain B given priority over strain A and measuring the resultant growth of each would allow these questions to be addressed. It was hypothesised that earlier arrival would give an advantage resulting in greater measured cell numbers, as the first isolate has possibly altered the environment for later arrivals.

- What growth factors are important for determining whether priority effects play a role? Microbial species all have different growth parameters which allow them to thrive under various environmental conditions. Parameters such as pH, Fe concentration and organics availability are all essential for growth of microorganisms, and as different species have different requirements for growth, these parameters can act to select for the successful coloniser to a new environment depending on limits or availability, and tolerance can be used to model microbial growth of different species [211],

[64], [223], [72], [48], [19], [10], [7]. The working hypothesis is that species with a broad range of pH tolerance and a low threshold for Fe and organics concentration necessary for growth would have higher growth rates and hence be more successful competitors in a variety of environments. If the environmental and nutritional needs of both species are met, and they have similar growth rates, priority effects are hypothesised to play a role in establishing which strain can grow to the highest concentration. If one species is better adapted or tolerant to the environmental conditions and nutritional parameters, or if the growth rates of the species are very different, or if the amount of time that one species is given priority is short compared to the growth rates of the strains, then priority effects are hypothesised to play a smaller role, or be insignificant to the growth achieved by both species.

5.3 Laboratory Methodology

The set-up of this experiment consists of various stages of preparation, each of which needed perfecting before proceeding to the next phase.

5.3.1 Pilot

An initial pilot study was conducted using coloured isolates from an existing experiment on "uninhabited habitats" [53] (an experiment that was not a part of this body of work), in order to achieve familiarity with the process of culturing and isolating coloured colonies on agar plates. These organisms were isolated from the atmosphere on sintered disks soaked in yeast extract (disks were soaked in 0.2% yeast extract solution and then dried in a laminar flow hood, for full protocol see [53]) and then grown on 0.2% yeast extract agar plates. Several transfers were conducted to ensure pure colonies that retained their initial colour over time on the solid medium.

5.3.2 Isolation of selected organisms

After the pilot study, strains of bacteria of different colours were isolated using yeast extract plates in an existing colonisation experiment, as described in

Chapter 3, as these colonisers were already of interest for further study. The existing colonisation experiment was set up in order to study the process of natural microbial colonisation of pristine, sterile rock microcosms through wind and rain. One of the questions under consideration in that study was the level at which community assembly is deterministic or stochastic, and whether each microcosms would host the same community over 18 months, or whether priority effects would play a role in assembling the community. If priority effects were of great importance, it is possible that identical microcosms would experience community divergence as a result. These observations come with the caveat that community divergence could also be a result of different seeding, if the input communities were not identical in all microcosms, and here the homogeneity of rain and wind is not known on sub-meter scales. In other words, if community divergence or differences are found between identical environments in the colonisation experiment, priority effects is one factor that could be responsible for this outcome. In order to be able to study the effect of priority in a more constrained environment, the experiment discussed in this chapter was set up, using isolates from the colonisation experiment. By using isolates from an existing experiment where priority effects may be playing a role, one would potentially be able to make inferences related to the process of community assembly that were relevant to both experiments.

The isolates were taken from existing 0.2% yeast extract agar plates from the colonisation experiment (Chapter 6) and chosen for their distinctive colour, from the set of colonies that were growing on the plates. These isolates were then re-plated several times on plates of various defined media, until a set was found to grow on M9 plates supplemented with an Fe source (for details of medium selection, see Section 5.3.3). In total, 14 strains were chosen from samples that had been collected after one, two and three months of colonisation in rock microcosms with crushed rock of either granite or gabbro, as seen in Figure 5.1. These 14 strains were chosen for their vibrant, distinct colouring observed while growing on agar plates, being favoured over strains with less distinct colours. Additionally, several transfers were conducted on several further colonies from one agar plate to the next, to ensure a pure strain, and these 14 were chosen from those that exhibited good consistency of growth appearance and morphology across these transfers.

Name	Colony colour	Months of colonisation before isolation
iso01	red	TP1
iso02	red	TP1
iso03	pink/red	TP1
iso04	orange	TP1
iso05	orange	TP2
iso06	orange/yellow	TP1
iso07	yellow tranparent	TP3
iso08	yellow light	TP3
iso09	yellow hard colonies	TP3
iso10	clear	TP3
iso11	clear	TP3
iso12	clear	TP3
iso13	white	TP3
iso14	white	TP3

Figure 5.1 *Initial coloured isolates from rock microcosms, with indication of the number of months of colonisation before culturing. TP=timepoint, indicates the number of months of colonisation before sampling, such that TP1=1 month, TP2=2 months, TP3=3months.*

5.3.3 Growth medium

It was necessary to find a suitable growth medium that would fit the further experimental set-up and aims, as the strains had been isolated on an undefined yeast extract agar, but as part of the long-term goal of the experiment the ability to control Fe concentration, organics concentration and pH was needed, in order to tie in the experiment with a mathematical model. The atmospheric strains have been isolated using culturing on yeast extract plates, which is problematic for determining bacterial growth functions, as it is a complex medium consisting of an undefined nutrient source, which, while providing for all the requirements of the organisms, makes it impossible to reliably alter the concentration of a particular nutrient. Hence, a defined medium on which the isolates would grow was needed. To this end, trials were conducted by growing the atmospheric isolates on glucose plates made from M9 medium, and glucose plates made from an unnamed, defined minimal weathering medium used in a weathering experiment (Chapter 6, adapted from [280]). As many of the isolates grew very poorly or not at all on these two substrates, another attempt was made to grow the isolates on M9 plates, this time supplemented with a Fe source, which resulted in 14 isolates growing successfully, as described in 5.3.2. This M9 medium with supplemented Fe was settled on as it is a defined medium, where it would be possible to alter the composition in terms of the concentration of supplied organics (glucose) and

Fe. Fe was added to the initial medium in a small amount as M9 on its own does not contain a Fe source, and no growth of the chosen environmental isolates was observed on M9 plates without supplied Fe. The final, unnamed supplemented M9 medium recipe can be found in Table 5.1.

Table 5.1 *M9 recipe overview*

Reagent	Amount
M9 salts	200ml
1M $MgSO_4$	2ml
20% glucose (liquid)	20ml
1M $CaCl_2$	100μl
$FeCl_3$ (anhydrous) at 252mg/l	10ml
MQ Water	770ml
Total volume	1l

Table 5.2 *M9 salts composition (x5)*

Reagent	Amount
Na_2HPO_4-$7H_2O$	64g
KH_2PO_4	15g
NaCl	2.5g
NH_4Cl	5.0g
MQ Water	make up to 1l
Total volume	1l

Due to the nature of the components of M9 medium, precipitation of calcium sulfate during autoclaving tends to occur if all components are added together simultaneously, and thus a special method needs to be employed to mix the ingredients together in order to ensure a sterile final product with no adverse chemical reactions having taken place. As such, first the M9 salts are made up (see Table 5.2) and autoclaved at a x5 concentration. Three separate bottles of 1M $MgSO_4$, 1M $CaCl_2$ and 20% glucose in MQ water are also made up and autoclaved. For the Fe supplement, a bottle of $FeCl_3$ (anhydrous) is made up at a concentration of 252mg/l and not autoclaved, hence needing to be filter sterilised into the final medium. In order to make up the final medium, a bottle of 770ml of MilliQ water is autoclaved, with 20g agar added before autoclaving if solid medium for agar plates is desired. Once autoclaved (and before cooling if making solid medium) the various components are added: M9 salts, $MgSO_4$, $CaCl_2$, and 20% glucose to a final concentration of 0.4%. Lastly, the non-sterile $FeCl_3$ mixture is added by filter sterilisation, by taking up the solution in a syringe and passing it through a 0.22μm filter (Nalgene) as it was added to the medium.

5.3.4 Transfers and final isolate selection

In order to ensure that the selected isolates would behave as necessary for the study in that they could be successfully cultured on both solid and liquid media, several transfers were made from old to new agar plates. The first aim of the transfers was to establish that the isolated strains were pure, and that no other colonies were cultured alongside the desired strain. If this had been the case, growth of several types of colonies would be observed on the agar plates, and with each transfer the likelihood of having contaminants transferred goes down. The second aim of the transfer was to ensure that each strain stayed the same in terms of morphology and colour, and that no unexpected alterations occurred after several transfers. The third aim was to establish that the strains could grow successfully in both liquid and solid medium, and be transferred back and forth between them. Thus, transfers were also conducted between solid and liquid media, and from liquid cultures into fresh liquid medium. After some time, four isolates of different colours were selected for the final experiment, as they were deemed the most reliable strains to work with, namely iso01 red, iso04 orange, iso08 yellow and iso13 white, seen in Figure 5.2.

Name	Colony colour	Months of colonisation before isolation
iso01	red	TP1
iso04	orange	TP1
iso08	yellow light	TP3
iso13	white	TP3

Figure 5.2 *Final four coloured isolates from rock microcosms, with indication of the number of months of colonisation before culturing.*

5.3.5 Pilot runs

Several iterations of this experiment were piloted in order to test the methodology and approach. This included trialling different isolates, isolate combinations and media, and ensuring that different coloured colonies were distinguishable if plated out from a liquid co-culture onto the same agar plate.

5.3.6 Methods: First mixing experiment

An initial mixing experiment was conducted using two of the selected isolates, iso08 yellow and iso13 white. Cultures were set up in 50ml Falcon tubes from transfers of cells from established liquid cultures. Firstly, 20ml of M9 medium was added to the 50ml tubes. Secondly, 200μl of various inocula was added to triplicate tubes in the following sample set-up: i) white, ii) white, iii) yellow, iv) yellow, v) white + yellow, vi) negative control. Sample v) had both strains added at the same time, in order to investigate how the strains grew together without priority effects. Samples ii) and iv) were to be kept as monocultures, while samples i) and iii) were to have the other strain added to them half-way through the experiment. Thus, samples i) and iii) were the ones where priority effects were tested, while ii), iv) and v) were different positive controls, and iv) the negative control. The samples were then incubated on the bench at ambient temperature (21°C). After two days, the samples were serially diluted and plated out on M9 plates. Immediately after this was added 200μl from cultures iv) in triplicate to i) triplicate samples, and from ii) in triplicate to iii) triplicate samples. The samples were again incubated on the bench, and after another two days plated out on M9 plates. At each time of plating, optical density (OD) was measured for all samples using a SpectroStar Nano spectrophotometer (BMG Labtech, Aylesbury, UK).

5.3.7 Methods: Second mixing experiment

After the first mixing experiment, another was set up, using the same two isolates. Some modifications were made in order to include more positive controls, as per the inoculation schedule in Figure 5.3, and to investigate whether some of the problems with clumping that had been observed previously would be repeated with smaller volumes. Thus, this time, the liquid amounts involved were slightly smaller, so cultures were set up in 15ml Falcon tubes. To these, 5ml of M9 medium to each 15ml tube was added. Then, 50μl of inoculum was added to the following tubes: i) white, ii) white, iii) Control, iv) yellow, v) yellow, vi) Control, vii) white + yellow, viii) negative control, as can be seen Figure 5.3. Sample vii) had both strains added at the same time, in order to investigate how the strains grew together without priority effects. Samples ii) and iv) were inoculated at the start and kept as monocultures, while samples iii) and vi) had one strain added

half-way through the experiment and remained as monocultures. Samples i) and iv) were to have the other strain added to them half-way through the experiment. Thus, samples i) and iv) were the ones where priority effects were tested, while ii), iii), v) and vi) were different positive controls, and viii) the negative control. These new cultures were plated out on M9 plates straight away at x1 (no dilution) concentration only, in order to enumerate cell counts. The cultures were left to incubate on the bench at 21°C. After three days of incubation, the samples were plated out on M9 plates. After this, 500μl of each of the white and yellow cultures was removed for adding to other culture (the white strain added to an already established culture of the yellow strain, and the yellow strain added to an already established culture of the white strain). Then, 50μl of opposite culture was added to the following tubes: i) yellow, iii) white, iv) white, vi) yellow. This was taken from the initial positive controls; take from v) to add to i) and vi), take from ii) to add to iii) and iv). After another three days the samples were plated out on M9 plates for enumeration of cells, and after another three days colonies of each colour was counted. The inoculation graphic can be seen in Figure 5.3. One improvement on the first mixing experiment is thus that there are extra positive controls added after three days, where the same aliquots that are added to the mixed samples are added to tubes with fresh medium, in order to control for the interim growth since the start of the experiment.

5.3.8 Methods: Third mixing experiment

The third mixing experiment was somewhat more of a proof-of-concept study, in order to investigate two suspected issues with the experiment. Firstly, there was some concern that there may be some contamination present in some of the strains. Secondly, there appeared to be some level of clumping in all strains when they were growing in liquid culture, as the CFU counts on the agar plates had a high variance, and also visible masses of cells were evidently moving in concert when the tubes were swirled around. In order to further investigate these issues, a simpler experiment was set up to see whether the results came out uncontaminated and whether the counts were reliable, indicating that clumping was not too much of an issue. Thus, the experiment was set up with smaller volumes in 6-well plates.

Two experiments were conducted on three isolates, by mixing iso08 yellow with iso04 orange, and iso01 red with iso04 orange. These new isolate combinations

		White+Yellow				Red+orange	
		Day 0	Half-way			Day 0	Half-way
	i	white	yellow			Red	Orange
	i	white	yellow			Red	Orange
	i	white	yellow			Red	Orange
	ii	white				Red	
	ii	white				Red	
	ii	white				Red	
	iii		white				Red
	iii		white				Red
	iii		white				Red
	iv	yellow	white			Orange	Red
	iv	yellow	white			Orange	Red
	iv	yellow	white			Orange	Red
	v	yellow				Orange	
	v	yellow				Orange	
	v	yellow				Orange	
	vi		yellow				Orange
	vi		yellow				Orange
	vi		yellow				Orange
	vii	y + w				r + o	
	vii	y + w				r + o	
	vii	y + w				r + o	
Control	viii						
	take from 5 to add to 1 and 6						
	take from 2 to add to 3 and 4						

Figure 5.3 *Inoculation schedule for the second and third mixing experiments, detailing the order in which the different coloured isolates were added to the samples.*

were chosen as there were concerns about clumping in the previous set, specifically the iso13 white strains, which was here interchanged for one red and one orange isolate. These new isolates were chosen as they appeared to have grown well on agar plates, and exhibited the least clumping in the liquid cultures of the remaining isolates under study. In addition, the colours were chosen such that there would be a visual difference between the existing yellow strain and the new orange and red strains, when cultured on agar plates.

The experiments follow the pattern of the two previous ones in that one isolate was given a week's priority before the other was added, and then the communities were left to incubate on the bench for another week before plating on M9 plates. Note that in this experiment no plating was conducted before mixing, but only at the end-point, in order to keep the design simple for testing the robustness of the methodology. Plating was done in triplicate on triplicate samples. In this study no positive controls were used in order to keep the number of samples down for this proof-of-concept experiment, so each 6-well plate contained initially three wells with each isolate, which were then cross-inoculated half-way through the study. In order to ensure that all samples could be mixed, an appropriate

aliquot was taken out of each culture before cross-inoculation. Slightly different sample volumes were used with the two different parts of this experiment. For the experiment of mixing the yellow and orange strains (iso08 yellow and iso04 orange), 5ml of medium was used in each well, and 50μl of inoculum culture was used. In the mixing study of red and orange (iso01 red and iso04 orange), 40μl of inoculum culture was used into 4ml of medium.

5.3.9 Growth curves

Growth curves for the different isolates were conducted according to two different protocols, one utilising 96-well plates in a plate reader, and one where the samples were grown in 50ml Falcon tubes, and sacrificial samples were taken every day on which OD was measured. Measuring the growth curves enabled inferences to be drawn as to how compatible the strains were in terms of growth rates, and whether growth rate differences can mask priority effects.

5.3.9.1 Plate reader growth curves

Plate reader growth curves were created using a SPECTROstar Nano absorbance plate reader (BMG LABTECH, Offenburg, Germany) at OD (optical density) 600nm. A series of pilot runs were conducted on the initial strains for this experiment that were growing on yeast extract. With the M9 isolates used for the main experiments presented here, two runs were conducted on the plate reader for each of iso01 red, iso04 orange, iso08 yellow, iso10 clear and iso13 white. For each strain, triplicate wells were prepared, with 200μl of medium and 10μl culture inoculum. Negative controls were used in triplicate. Note that the cultures were not normalised to the same OD or concentration before the start of the run, as the samples were tested at the same point in time rather than the same OD or concentration. The first run was conducted for 94.5h and the second for 74.5h, with the first run being longer than necessary.

5.3.9.2 Manual growth curves using Falcon tubes

Some problems were experienced with the plate reader growth curves, after which it was decided to attempt to do manual growth curves, coupled with CFU counts from plating at the same time points, in order to get accurate correspondence

between OD and cell counts for each strain. Cultures of iso01 red, iso04 orange, iso08 yellow, iso10 clear and iso13 white were set up in 50ml Falcon tubes, using 200μl of inoculum culture into 20ml of fresh M9 medium. These were incubated in the bench at ambient temperature (21°C) for four days. At 3pm each day, aliquots of 30μl were removed, to be used for plating, and serial dilution was conducted down to x10^3 initially and x10^6 for the last time point (after four days). After plating, 1ml of culture was removed from each sample and placed in a cuvette, and OD measured at 600nm using the cuvette port on a SPECTROstar Nano absorbance plate reader (BMG LABTECH, Offenburg, Germany).

5.3.10 Results

5.3.11 Results: First mixing experiment

The first mixing experiment yielded some useful insights, as it enabled the comparison of growth of two different coloured isolates when either one was given priority in colonising a new environment. The data are presented in Figure 5.4.

This shows that the final community is dominated by whichever isolate was the primary coloniser. In the case where white is added first, the yellow strain makes up about 20% of the end-point community. When yellow is added first, the white strain makes up a negligible part of the final community. The difference in the growth of the white strain when it is added first or second is statistically significant at the 0.05 confidence level (t-test, p=0.000106), while the difference in growth for the yellow strain is not (t-test, p=0.115), although the average growth is lower when yellow is added last compared to when it is added first. This supports the idea that priority effects can occur (white) but that high variation means that in some cases although the averages are different the results may not be statistically significant at a low level. The white strain shows the largest change in growth depending on whether or not it is added first. Differences in growth rates between the strains cannot explain these results, as evidenced by the positive controls (samples ii for white and samples iv for yellow).

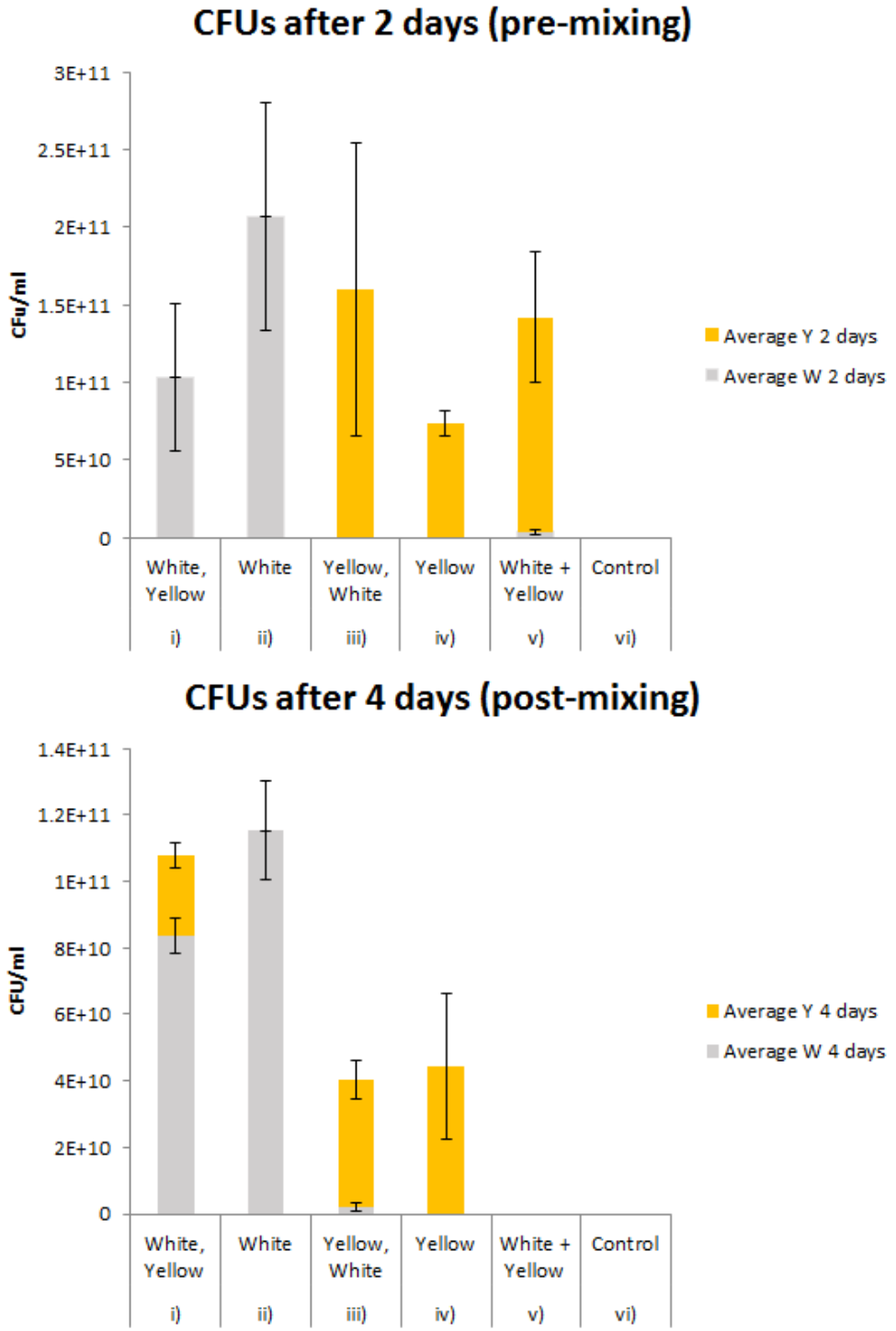


Figure 5.4 *CFUs/ml for the first mixing experiment, measuring viability on M9 plates of the two different colony types after 2 days (pre-mixing) and 4 days (2 days post-mixing). Y=yellow, W=white. Error bars are standard error (n=3).*

5.3.12 Results: Second mixing experiment

The results from the second mixing experiment corroborate those from the first (Figure 5.5). Again, it is found that the primary isolate comes to dominate the

final community. The white strain shows the largest change in growth depending on whether or not it is added first. The differences in growth rate for the yellow and the white isolates depending on whether they are added first or last are not statistically significant at the 0.05 confidence level, as the variance is large even though the averages change. Thus, the experiment shows priority effects can occur, but that variation is high and often results in a statistically insignificant difference although averages remain different. When both isolates are added at the same time (sample vii), the final community is dominated by the white isolate, but after three days the community is relatively evenly distributed in terms of CFUs. Notably, the white isolates appear to grow better in the presence of the yellow isolate than without it, as evidenced by the positive controls. Conversely, the yellow isolate may grow better alone than in the presence of the white isolate.

The results seem to indicate that priority effects do play a role, but the effect was somewhat unclear due to the variance in the CFU counts between triplicates. This was suspected to be a result of the clumping and thus the liquid may not have been properly homogenised before aliquots were removed for plating, even though every effort was made to homogenise the liquid using standard pipetting techniques and shaking the tubes. It was evident that the cells grew as a biofilm clump in the liquid, so from a visual inspection it seemed as if the homogenisation efforts were not completely effective. It was thus decided to try another experiment, with a simpler design, fewer replicates and using other isolate strains, to see whether the problems with clumping could be circumvented, or would persist with an altered set-up.

5.3.13 Results: Third mixing experiment

The results from the third mixing experiment suggest that there is no effect of priority on the final community, as the end-point communities show little variation, for both isolate combinations (Figures 5.6 and 5.7). This stands in contrast with the previous experiments, although these are using a different isolate set compared to the previous two experiments. This new set of isolates was settled upon as there were concerns about clumping and heterogeneity in the growth of especially the white isolate, which would affect CFU count reliability.

The results from the third mixing experiment show that one strain consistently ends up dominating the final community, regardless of whether it was added first or last. In the combination of the orange and yellow strains, the yellow strain

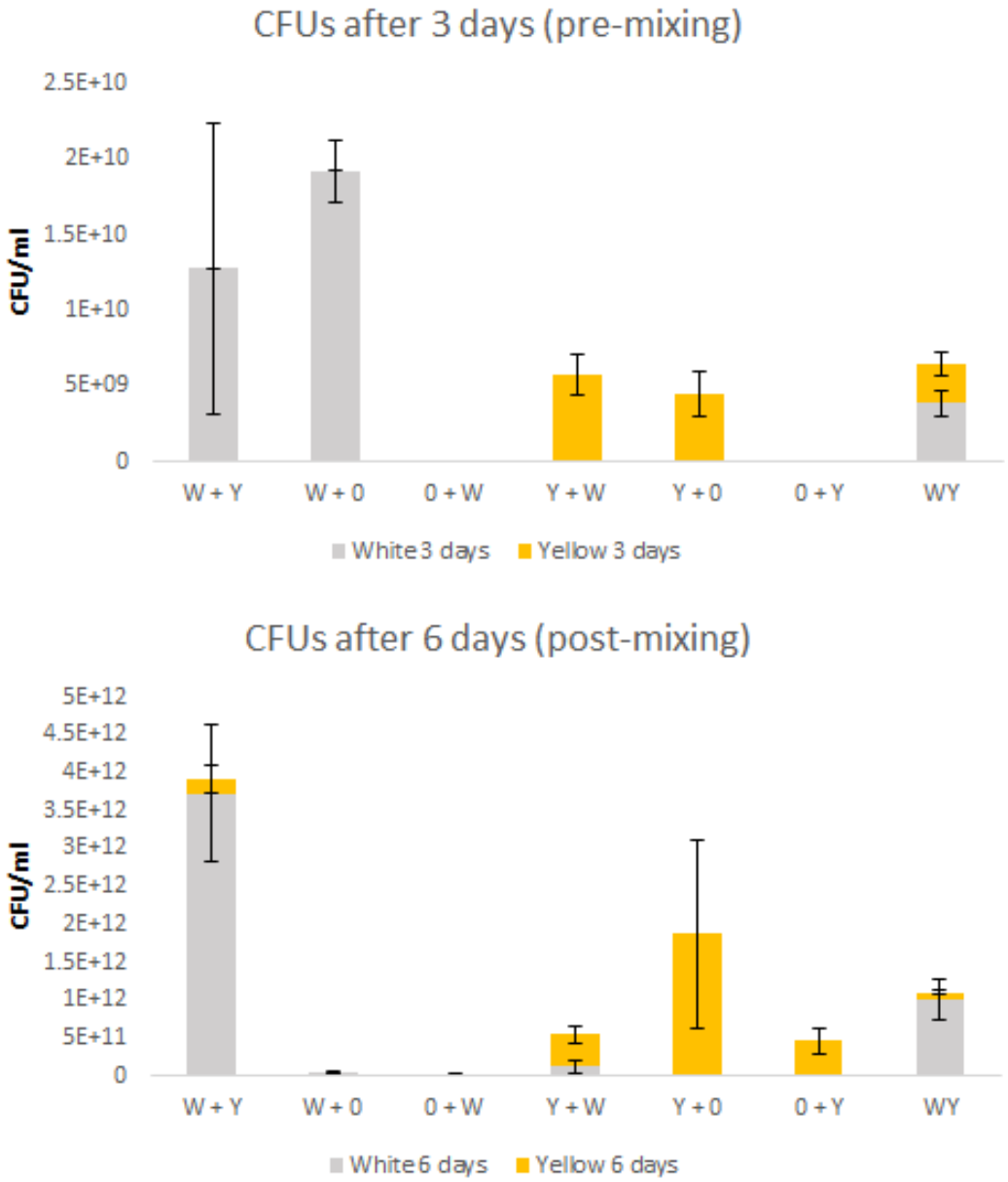


Figure 5.5 *CFUs/ml for the second mixing experiment, measuring viability on M9 plates of the two different colony types after 3 days (pre-mixing) and 6 days (3 days post-mixing). Y=yellow, W=white. Error bars are standard error (n=3).*

ends up dominating the community after two weeks, regardless of whether it was added first or second, i.e., whether it was added as the first strain at time zero, or half-way through after 1 week, when the orange strain had already had 1 week to grow in the medium. Likewise, the same effect was found for the combination of adding the orange and red strains together, but here the orange strain ends up dominating the end-point communities, regardless of whether it was added first or

last. This means that for these two combinations of isolates, priority effects do not matter, as one strain consistently ends up dominating the end-point community. Had priority effects played a role, whichever isolate had been added first would have been expected to dominate the final community. The fact that the same strain ends up being more successful in terms of final amounts of growth suggests that here, priority effects may be giving way to other factors, such as differences in growth rates or requirements between the strains under study.

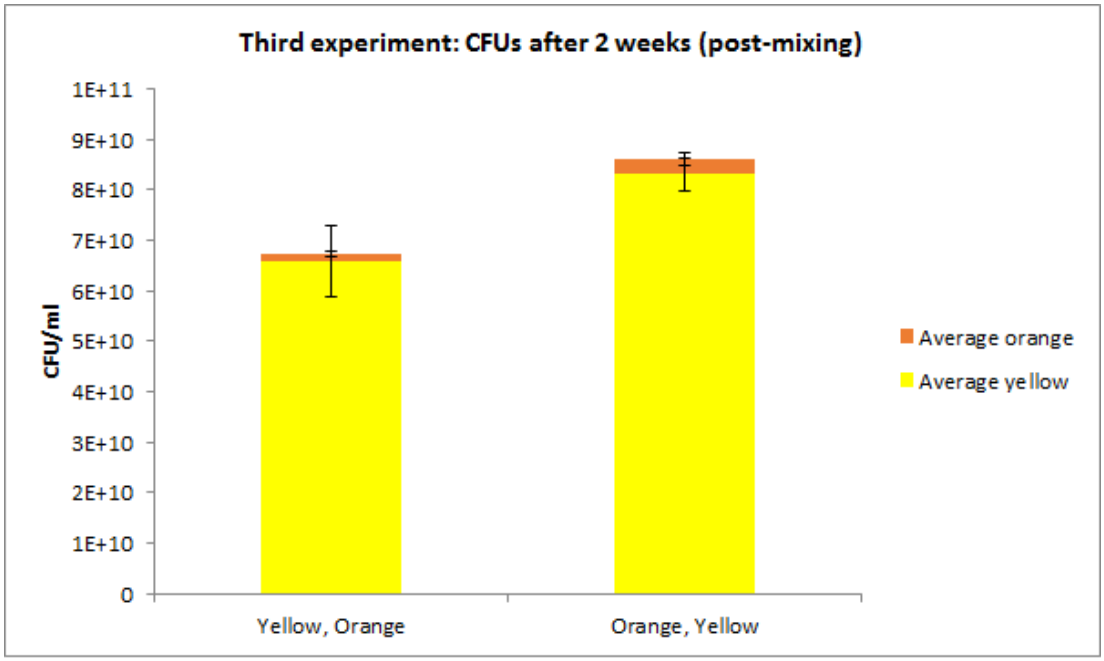


Figure 5.6 *CFUs/ml for the third mixing experiment, measuring viability on M9 plates of the two different colony types yellow and orange after 2 weeks (1 week post-mixing). Error bars are standard error, n=9.*

5.3.14 Concerns about clumping in liquid culture

Some of the work undertaken in this study have indicated the possibility of clumping and hence heterogeneity in cell concentration in liquid culture, affecting the CFU counts on agar plates that were used to enumerate growth. Visual inspection of the liquid cultures showed that all isolates under study were showing various levels of cells clumping together in the flask, such that when swirled the biomass moved as one. Some of the results listed in Figures 5.8 and 5.9 show that triplicate samples have very varied cell counts, and that dilution series are not exponentially following the factor of dilution. Repeated attempts were made to find a way to homogenise the liquid cultures before aliquots were removed for

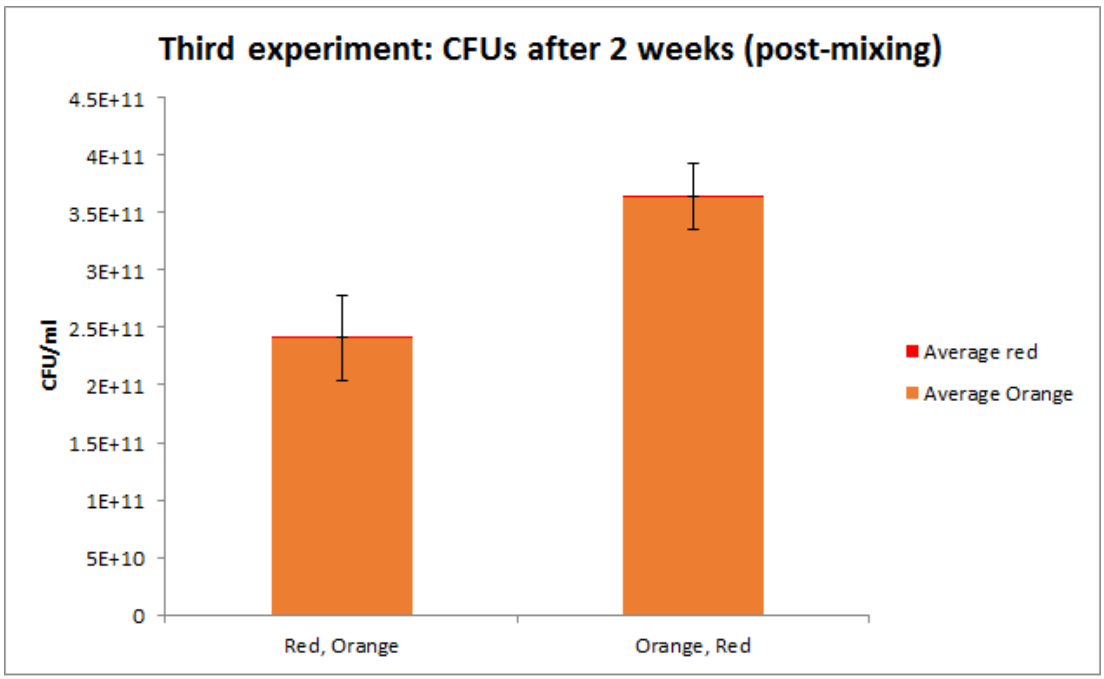


Figure 5.7 *CFUs/ml for the third mixing experiment, measuring viability on M9 plates of the two different colony types red and orange after 2 weeks (1 week post-mixing). Error bars are standard error, n=9.*

plating, however, mixing never appeared to be completely successful, even when pipettes were used to draw the liquid up and down for several minutes. Taken together, it was deemed that cell counts in continued experiments might not be reliable, which was crucial for the success of the study.

5.3.15 Growth curve results

5.3.15.1 Plate reader growth curve results

With the M9 isolates used for the main experiments presented here, two runs were conducted on the plate reader. Details for iso08 yellow and iso13 white, which were the strains used in the first and second mixing experiments, are found in Figure 5.10. The growth curves show a significant amount of noise and fluctuations, which may be due to the strains not growing homogeneously in space and/or time in the wells. As the growth curves were running for 94h, it is also possible that evaporation may affect readings towards the end of the time series. Figure 5.11 shows the growth curves in M9 for the 13 isolates over 94h.

yellow, then orange							
	Dilution	CFUs a	CFUs b	CFUs c	Average	Triplicate average CFUs	
Well 1	10^4	2	0	0	0.666667		
	10^6	0	0	0	0	10^4	3.666667
Well 2	10^4	1	5	6	4	10^6	1.555556
	10^6	0	4	1	1.666667		
Well 3	10^4	3	11	5	6.333333		
	10^6	5	1	3	3		
orange, then yellow							
	Dilution	CFUs a	CFUs b	CFUs c	Average		
Well 1	10^4	0	2	1	1		
	10^6	0	0	0	0	10^4	3.888889
Well 2	10^4	0	4	4	2.666667	10^6	2.888889
	10^6	2	1	1	1.333333		
Well 3	10^4	1	9	14	8		
	10^6	3	8	11	7.333333		

Figure 5.8 *CFU counts for the third mixing experiment, measuring viability on M9 plates of the two different colony types yellow and orange after 2 weeks (1 week post-mixing). In one condition, yellow was given one week's priority before orange was added, and in the other condition orange was given one week's priority before yellow was added. The samples were plated after 2 weeks in total, and counts measured. Here, the counts at 10^4 are presented as a comparison to the 10^6, indicating that no ten-fold dilution is apparent in the cell counts, which may be due to problems with clumping.*

red, then orange							
	Dilution	CFUs a	CFUs b	CFUs c	Average	triplicate average CFUs	
Well 1	10^2	22	25	18	21.66667	10^2	35.33333
	10^4	0	0	2	0.666667	10^4	0.666667
Well 2	10^2	30	34	58	40.66667		
	10^4	1	1	1	1		
Well 3	10^2	57	42	32	43.66667		
	10^4	0	0	1	0.333333		
orange, then red							
	Dilution	CFUs a	CFUs b	CFUs c	Average	triplicate average CFUs	
Well 1	10^2	148	too much	too much	148	10^2	49.33333
	10^4	3	4	3	3.333333	10^4	1.111111
Well 2	10^2	0	0	0	0		
	10^4	0	0	0	0		
Well 3	10^2	0	0	0	0		
	10^4	0	0	0	0		

Figure 5.9 *CFU counts for the third mixing experiment, measuring viability on M9 plates of the two different colony types red and orange after 2 weeks (1 week post-mixing). In one condition, red was given one week's priority before orange was added, and in the other condition orange was given one week's priority before red was added. The samples were plated after 2 weeks in total, and counts measured. Here, the counts at 10^4 are presented as a comparison to the 10^2, indicating that no ten-fold dilution is apparent in the cell counts, which may be due to problems with clumping.*

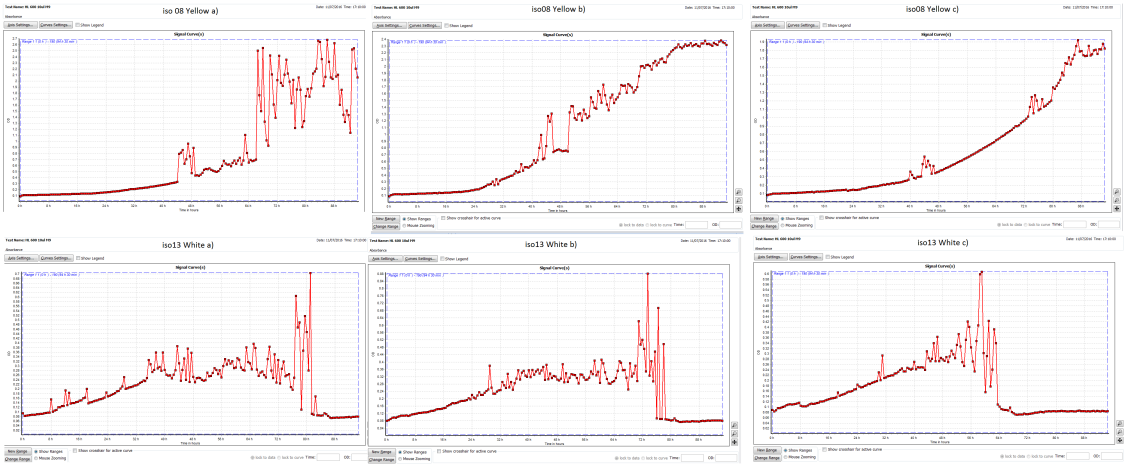


Figure 5.10 *M9 growth curves for iso08 yellow and iso13 white in triplicate. Growth curves were measured at OD 600nm, every 30mins for 94.5 hours, in 96-well plates using a plate reader.*

5.3.15.2 Manual growth curve results

The manual growth curves from growing larger volume cultures in 50ml Falcon tubes are presented in Figure 5.12. The different strains can be seen to have different growth rates, with iso10 clear achieving the highest OD readings over the four days. The strains used for the first two mixing experiments, iso08 yellow and iso13 white are found to have similar values of absorbance at the points of measurement, as does their composite mixed culture. The strains used for the third mixing experiment, iso01 red and iso04 orange and their mixed culture have similar, low absorbance readings at the different time points of measurement.

5.4 Isolates model

5.4.1 Introduction

There are many instances where mathematical models have been used to describe complex microbial systems in various environments. The subject benefits from a multi-method approach including both field studies, laboratory enrichments and modelling. In particular, laboratory experiments may overlook species whose niche is not included in the laboratory simulation or replication of the natural environment, as laboratory conditions are often more constrained and controlled than real environments [259], [231]. Modelling enables simplification

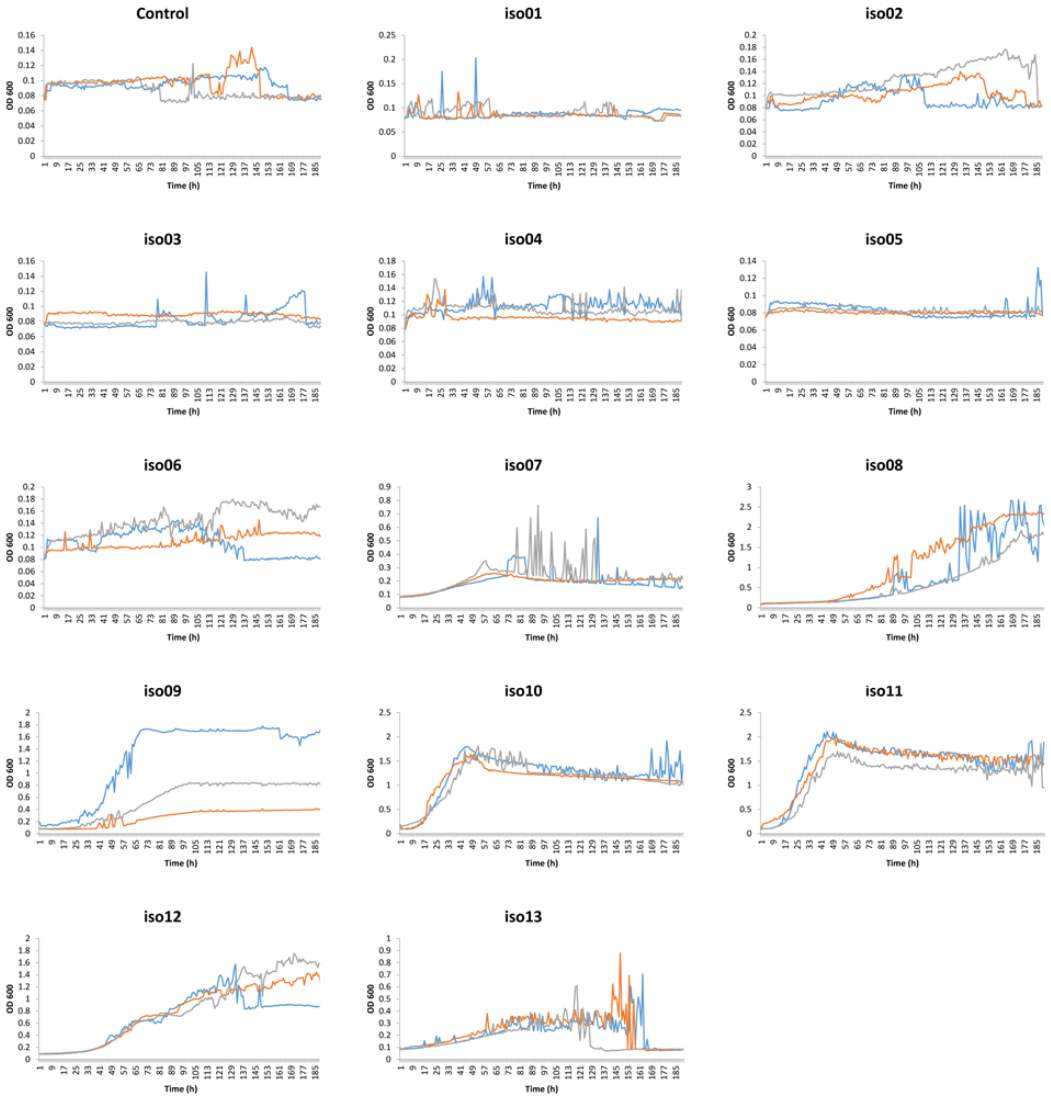


Figure 5.11 *M9 growth curves for all 13 isolates in triplicate. Growth curves were measured at OD 600nm, every 30mins for 94.5 hours, in 96-well plates using a plate reader. Each graph shows triplicate samples as different lines.*

of an otherwise highly diverse system, especially if functional redundancy can be accounted for [149].

A model was conceptualised that would mirror the experimental set-up in the isolates experiment, again with the aim of studying the factors involved in the early stages of community assembly. The aim of the model was to investigate whether priority effects and the results observed in the laboratory could be replicated using the differences in growth curves and requirements of the studied species. As such, the goal was to measure the relevant parameters of growth

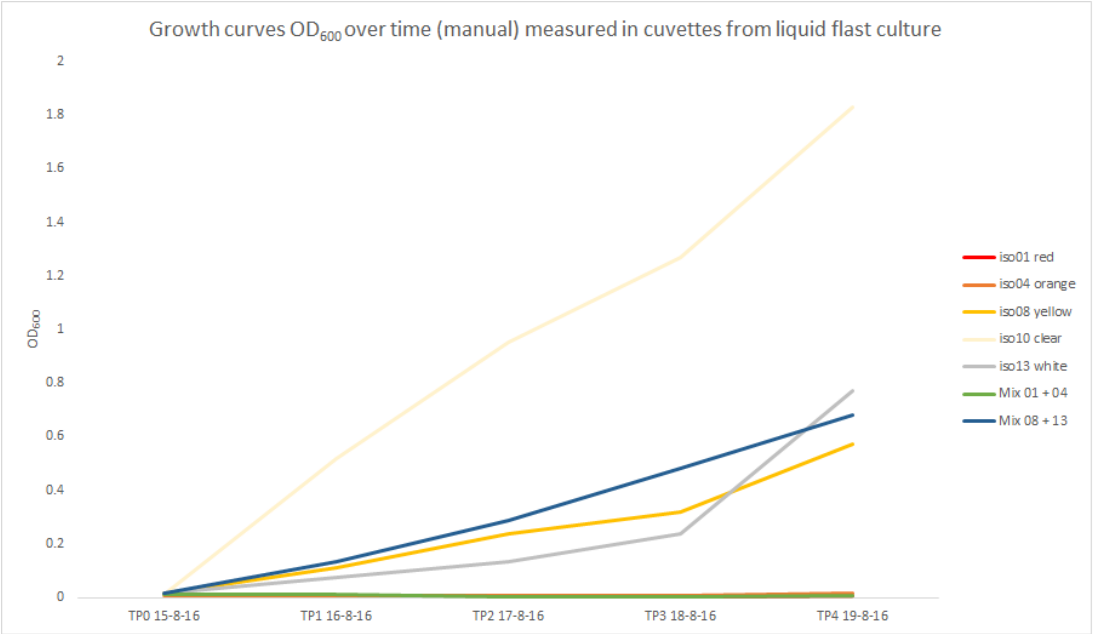


Figure 5.12 *M9 growth curves for selected strains used in isolates mixing experiments. Growth curves were measured at OD 600nm using 1ml of liquid culture in a cuvette. Cultures were grown in 50ml Falcon tubes, and 1ml aliquots removed each day for four days in order to measure growth.*

of the selected isolates in the lab, such as growth rate, pH tolerance, organics requirement and Fe requirement, and then feed these into the model, to simulate the interaction *in silico*, and investigate whether key parameters involved in producing the laboratory results could be identified. The model is based on Monod kinetics of bacterial growth, and takes into account how the concentration of organics and Fe affect growth of a specific strain. The model has two organisms with different growth characteristics which can be added to the system at different times. A sample plot is presented in Figure 5.13, where one strain starts growing at t=0, while the other strain is introduced half-way through, resulting in an increased drop in the organics concentration.

5.4.2 Motivations and hypotheses

The motivation for creating a model was to be able to simulate the laboratory observations, in order to be able to determine whether the key variables affecting the results in the lab could be narrowed down. The scientific questions under investigation here were related to those of the isolates experiment, focussing on the importance of priority effects in community assembly, and the factors

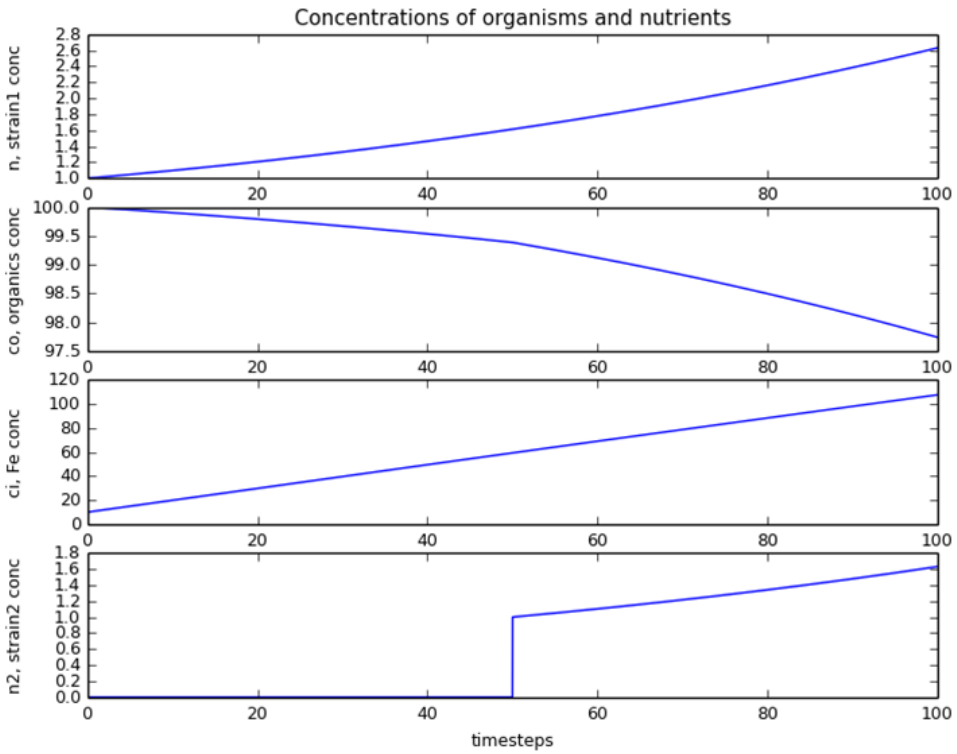


Figure 5.13 *Behaviour of the isolates model with the first strain starting growth at t=0 and the second strain added half-way through, with both strains having the same growth rate. From top to bottom the panels show: n1, the number of organisms of type 1; co, concentration of organics; ci, concentration of Fe; n2 number of organisms of type 2.*

which determine the influence of priority on the final community. The model was constructed alongside the experiment with the aim of testing whether the results in the experiment could be replicated using a known set of growth characteristics for the strains used in the laboratory. Focus was placed on growth rates, time allotted for priority, and three environmental variables commonly affecting growth of microorganisms: pH tolerance, organics requirements and Fe requirements. These are all environmentally relevant variables that characterise growth, and are relatively easy to control in laboratory experiments.

5.4.3 Modelling methodology

The model is based on Monod kinetics of bacterial growth [182].

The key equation when modelling microbial growth is a differential equation

expressing the rate of change of the population:

$$\frac{dn}{dt} = \text{growth} - \text{death} \tag{5.1}$$

where n = number of microbes or concentration (number density) and t = time. The death rate is modelled by some constant rate D multiplied by n, hence death $= Dn$. The growth rate is assumed to follow the Monod equation [182], which is $\frac{VS}{K+S}$, where V = specific growth rate [time^{-1}], S = concentration of the substrate and K is the value of S at $V/2$. Because V refers to the specific growth rate, the term $\frac{VS}{K+S}$ describes the growth of a single individual, hence the term is multiplied by n. This gives:

$$\frac{dn}{dt} = \frac{VS}{K+S}n - Dn \tag{5.2}$$

There are several alternative formulation to Monod kinetics, amongst others the Contois method [62], for modelling microbial growth, but Monod is by far the most prevalent method. Values for constants V and K can be found in the literature for many common organisms on different substrates [221], [180], [119].

The concentrations of substrates under consideration can be modelled as follows, where y signifies the yield, which is how much unit substrate is needed to build one new unit of cell or organism:

$$\frac{dc}{dt} = -y \cdot V \cdot n \cdot \frac{c}{K+c} \tag{5.3}$$

If a substrate is being replenished into the system, this can be expressed using a rate constant r as follows:

$$\frac{dc}{dt} = -y \cdot V \cdot n \cdot \frac{c}{K+c} + r \tag{5.4}$$

To model microorganisms that are growing on two different substrates there are many different ways to describe the growth kinetics. Two of the most common approaches are the multiplicative method and Liebig's Law. The multiplicative model is expressed as [15]:

$$\frac{dn}{dt} = Vn\left(\frac{S_1}{K_1+S_1}\right)\left(\frac{S_2}{K_2+S_2}\right) - Dn \tag{5.5}$$

The multiplicative method thus multiplies the factors of the two substrates

together, so that the two substrates can be of varying importance at different times or combinations depending on the value of their growth parameters. A second method for treating two substrates is Liebig's Law of the Minimum, which is based on the idea that either substrate will be limiting at any one time, and thus only the parameters of whichever substrate is lowest is taken into account at every single point [201]:

$$\frac{dn}{dt} = Vn \times Min\left(\left(\frac{S_1}{K_1 + S_1}\right), \left(\frac{S_2}{K_2 + S_2}\right)\right) - Dn \tag{5.6}$$

pH tolerance can be modelled through a number of different ways. In the simplest form, pH is modelled as a range within which maximum growth is allowed, and outside which no growth takes place, which is the approach adopted here. For variations of this method, in general, the ends of the range can be adjusted to allow for some sub-maximum growth. A slightly more complicated variation is a trapezoid range, whereby the growth increases linearly from the outside of the pH tolerance range until maximum growth is achieved in the appropriate sub-range. Other methods have proposed, based on experimental data, that pH can be modelled as various forms of exponential functions or statistical physics approaches, whether symmetrical or not [158], [235].

Here, the model was based on the multiplicative approach of Monod kinetics, where the two growth parameters under consideration are the concentration of Fe and organics in the system. Fe is taken as a proxy for the main limiting nutrients, as it is the most essential nutrient that often ends up being limiting to growth [152]. At time t=0, finite amounts of Fe and organics are available to the system, with no replenishment, as could be the case in a closed microcosm if dead biomass did not resupply the system with nutrients. Two strains are present, which can have similar or different growth characteristics. The model allows for the addition of the strains at various times, such that one strain can be added from the beginning, and the other strain added at a later time. pH is initially set to a certain value, and then decreases with time. Ultimately, pH can be modelled to have some sort of dependence on the concentration of organics, as the consumption of organics would in a closed, unbuffered system lead to a build-up of acid, lowering the pH. Each strain is assigned a minimum and maximum pH for growth, between which maximum growth occurs, and outside which no growth occurs. The code can be seen in Listing 5.1.

Listing 5.1: Isolates model proof-of-concept code

```
import matplotlib.pyplot as plt # Import library that does maths.

# Declare variables in lists
t = [] # t = time
n = [] # n = number of organisms
co = [] # co = concentration of organics
ci = [] # ci = concentration of iron Fe
n2 = [] # n2 = number of organisms of species 2
ph = [] # ph = pH

# Adds the first value to each list, all variables that change
    ↪ during simulation
t.append(0.0)
n.append(1.0)
n2.append(0.0)
co.append(100.0)
ci.append(10.0)
ph.append(7.0)

# Declares values to all other variables (not changing during
    ↪ simulation)
nsteps = 10000 # nsteps = number of timesteps
dt = 0.01 # dt = size of timestep
v = 0.01 # v = specific growth rate [time^-1], max growth rate
v2 = 0.01 # specific growth rate for species 2
Ko = 1.0 # Ko = value of co at v/2 for organics, concentration at
    ↪ which growth is half max
Ko2 = 1.0 # Ko = value of co at v/2 for organics, concentration at
    ↪  which growth is half max, for species 2
yo = 1.0 # yo = yield for organics, how many units of organics it
    ↪ takes to build one new unit of microbe
Ki = 1.0 # Ki = value of co at v/2 for iron, concentration at
    ↪ which growth is half max
Ki2 = 1.0 # Ki = value of co at v/2 for iron, concentration at
    ↪ which growth is half max, for species 2
yi = 1.0 # yi = yield for iron, how many units of iron it takes to
    ↪  build one new unit of microbe
```

```
r = 1.0 # r = rate of input of iron
a = 2.0 # some constant relation between pH and co
phmin = 3.0 # sets the minimum allowed pH

# Declares values to pH tolerances of different strains
nphmin = 8.0 # Minimum pH at which strain 1 can grow
nphmax = 10.0 # Maximum pH at which strain 1 can grow
n2phmin = 2.0 # Minimum pH at which strain 2 can grow
n2phmax = 6.5 # Maximum pH at which strain 2 can grow

f = open('results.dat', 'w') # Opens a file, called 'results.dat'
    ↪ and states that it shall be written to. The f gives the file
    ↪  a name in the program.

# Start a for loop for a set number of timesteps. At each step the
    ↪  values in the lists are updated. Lists start at value zero
    ↪ so the first index into the lists must be [i-1]. The
    ↪ equations are differential equations that are solved, and
    ↪ the results stored, for each timestep.
for i in range (1, nsteps):
   t.append(t[i-1] + dt)

   co.append(co[i-1])
   ci.append(ci[i-1])

   if ph[i-1] > phmin:
       ph.append(ph[i-1] - dt * 0.1)
   else:
       ph.append(phmin)

   if nphmax > ph[i-1] > nphmin:
       n.append(n[i-1] + dt * n[i-1] * v * (co[i-1] / (Ko + co[i
           ↪ -1])) * (ci[i-1] / (Ki + ci[i-1])))
       co[i] = co[i] - dt * yo * n[i-1] * v * (co[i-1] / (Ko + co[i
           ↪ -1])) * (ci[i-1] / (Ki + ci[i-1]))
       ci[i] = ci[i] - dt * yi * n[i-1] * v * (co[i-1] / (Ko + co[i
           ↪ -1])) * (ci[i-1] / (Ki + ci[i-1]))
```

```
else:
    n.append(n[i-1])
#if i < 100:
   #n2.append(0.0)

if i < 5000:
  n2.append(0.0)
elif i == 5000:
  n2.append(1.0)
else:
   if n2phmax > ph[i-1] > n2phmin:
      n2.append(n2[i-1] + dt * n2[i-1] * v * (co[i-1] / (Ko + co
         ↪ [i-1])) * (ci[i-1] / (Ki + ci[i-1])))
      co[i] = co[i] - dt * yo * n2[i-1] * v2 * (co[i-1] / (Ko2 +
         ↪  co[i-1])) * (ci[i-1] / (Ki2 + ci[i-1]))
      ci[i] = ci[i] - dt * yi * n2[i-1] * v2 * (co[i-1] / (Ko2 +
         ↪  co[i-1])) * (ci[i-1] / (Ki2 + ci[i-1]))
   else:
      n2.append(n2[i-1])

#co.append(co[i-1] - dt * yo * n[i-1] * v * (co[i-1] / (Ko + co[
   ↪ i-1])) * (ci[i-1] / (Ki + ci[i-1])) - dt * yo * n2[i-1] *
   ↪  v2 * (co[i-1] / (Ko2 + co[i-1])) * (ci[i-1] / (Ki2 + ci[
   ↪ i-1])))
#co.append(co[i-1] - dt * yo * n[i-1] * v * (co[i-1] / (Ko + co[
   ↪ i-1])) - dt * yo * n2[i-1] * v2 * (co[i-1] / (Ko2 + co[i
   ↪ -1])))
#co.append(co[i-1] - dt * yo * n[i-1] * v * (co[i-1] / (Ko + co[
   ↪ i-1])) * (ci[i-1] / (Ki + ci[i-1])))
ci[i] = ci[i] + dt * r

#ci.append(10.0)
print "%f␣%f␣%f␣%f␣%f␣%f" % (t[i], n[i], co[i], ci[i], n2[i], ph
   ↪ [i]) # Prints values to terminal
#print "%f %f %f %f " % (t[i], n[i], co[i], ci[i]) # Prints
   ↪ values to terminal
f.write("%f␣%f␣%f␣%f␣%f␣%f␣\n" % (t[i], n[i], co[i], ci[i], n2[i
```

```
      ↪ ], ph[i])) # Writes values to file
   #f.write("%f %f %f %f \n" % (t[i], n[i], co[i], ci[i])) # Writes
      ↪  values to file

plt.rcParams.update({'font.size': 9})

# Plots a figure with three subplots to show growth, concentration
   ↪  of organics and concentration of iron.
plt.figure(1) # Opens the figure
plt.subplot(511) # Creates the first subplot
plt.plot(t,n) # Plots n vs time
plt.xlabel('timesteps')
plt.ylabel('n, strain1 conc')
plt.title('Concentrations of organisms and nutrients')
plt.subplot(512) # Creates the second subplot
plt.plot(t,co) # Plots co vs time
plt.xlabel('timesteps')
plt.ylabel('co, organics conc')
plt.subplot(513) # Creates third subplot
plt.plot(t,ci) # Plots ci vs time
plt.xlabel('timesteps')
plt.ylabel('ci, Fe conc')
plt.subplot(514) # Creates fourth subplot
plt.plot(t,n2) # Plots n2 vs time
plt.xlabel('timesteps')
plt.ylabel('n2, strain2 conc')
plt.subplot(515) # Creates fifth subplot
plt.plot(t,ph) # Plots pH vs time
plt.xlabel('timesteps')
plt.ylabel('pH')
plt.show() # Shows the plot
```

5.4.4 Modelling results

The model generated interesting results for the two-species system. Figure 5.14 illustrates the behaviour of the system with only one species and no addition of

organics or Fe beyond what was initially provided. This system shows that the microbial population size, in the top panel, increases exponentially while Fe is present, while linear growth takes over once Fe is no longer available, and growth stops completely once the organics run out at about timestep 9000.

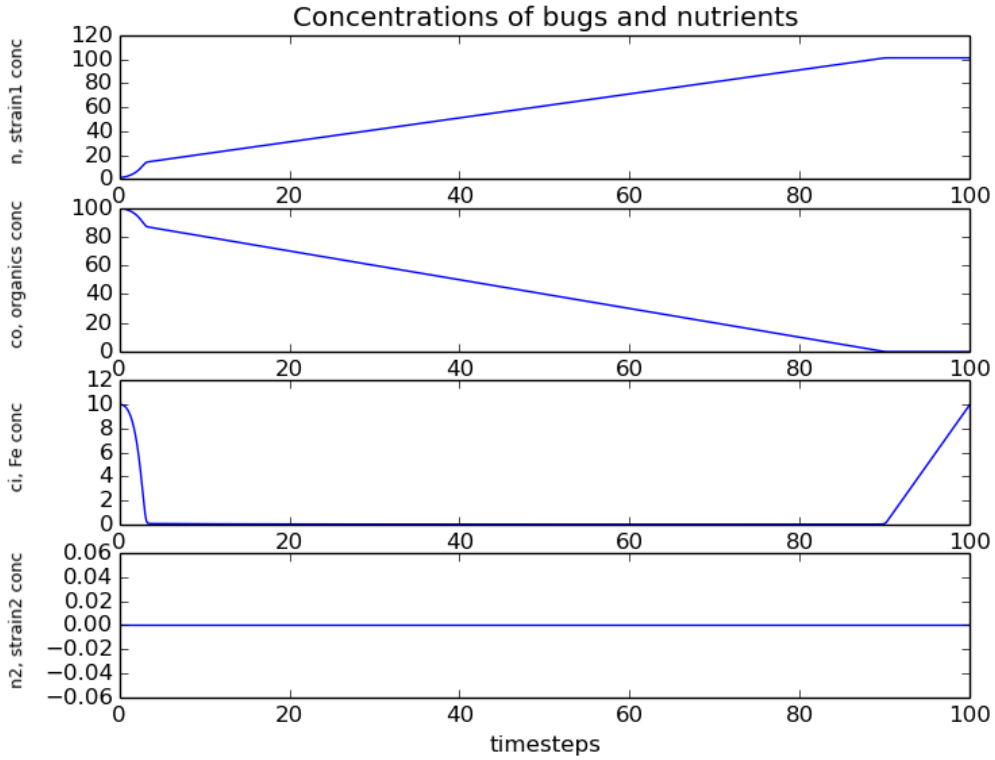


Figure 5.14 *Behaviour of the isolates model in its simplest iteration, with only one strain, and no addition of Fe or organics beyond the initial supplement. From top to bottom the panels show: n1, the number of organisms of type 1; co, concentration of organics; ci, concentration of Fe; n2 number of organisms of type 2.*

Figure 5.15 shows two examples of what happens when the second strain is added, if the growth rates and growth yields of both strains are equal. In the left hand graph, the second strain is added at 100 timesteps, while on the right hand side it is added after 5000 timesteps, which is halfway through the simulation run. If added at 100 timesteps, the second strain it is added after Fe has already run out and hence it does not grow to create another organism.

If there is no Fe limitation, both strains can grow exponentially until the organics run out, as evidenced by Figure 5.16.

Figure 5.17 shows the effect of adding a second strain while the first strain is

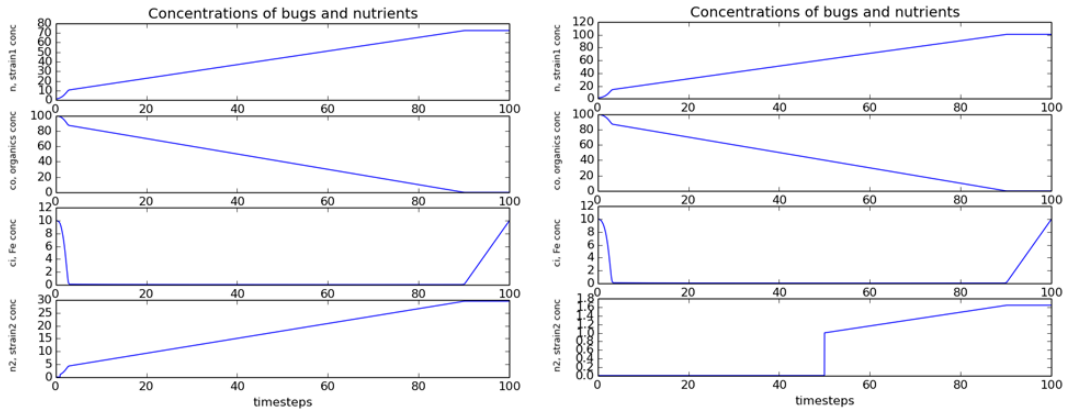


Figure 5.15 *Behaviour of the isolates model with no addition of Fe or organics beyond the initial supplement, with the second strain added after left) 100 timesteps and right) 5000 timesteps. From top to bottom the panels show: n1, the number of organisms of type 1; co, concentration of organics; ci, concentration of Fe; n2 number of organisms of type 2.*

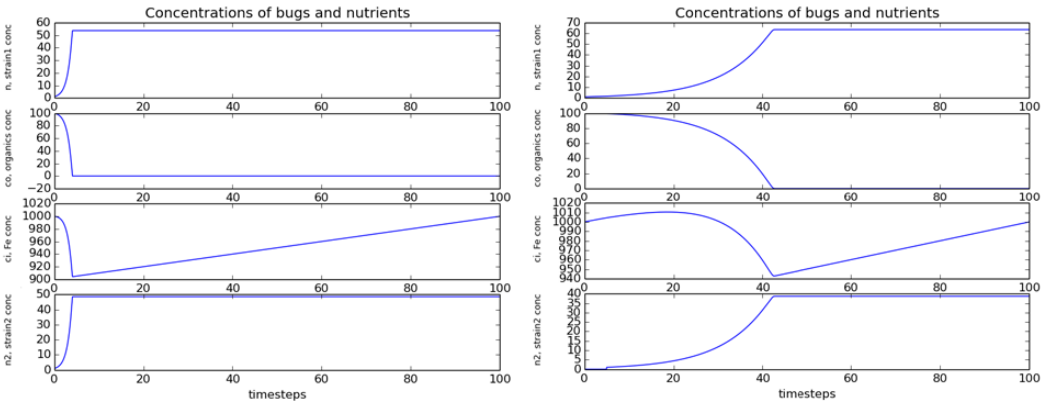


Figure 5.16 *Behaviour of the isolates model with no limitation of Fe, but where organics is not re-supplied. The second strain is added after left) 10 timesteps and right) 500 timesteps. The specific growth rates are left) v=1.0 for both strains and right) have been reduced to v=0.1 for both strains. From top to bottom the panels show: n1, the number of organisms of type 1; co, concentration of organics; ci, concentration of Fe; n2 number of organisms of type 2.*

in the early stages of exponential phase. Here, the specific growth rates of both strains are set to v=0.01 t^{-1}, and the second strain is added at timesteps a) not added, b) 1000, c) 5000 and d) 7000.

Some results can be seen in Figure 5.18 of the effects of changing the Fe supply rate. The various values of r are a) 0.1, b) 2, c) 10 and d) 100, where r is a

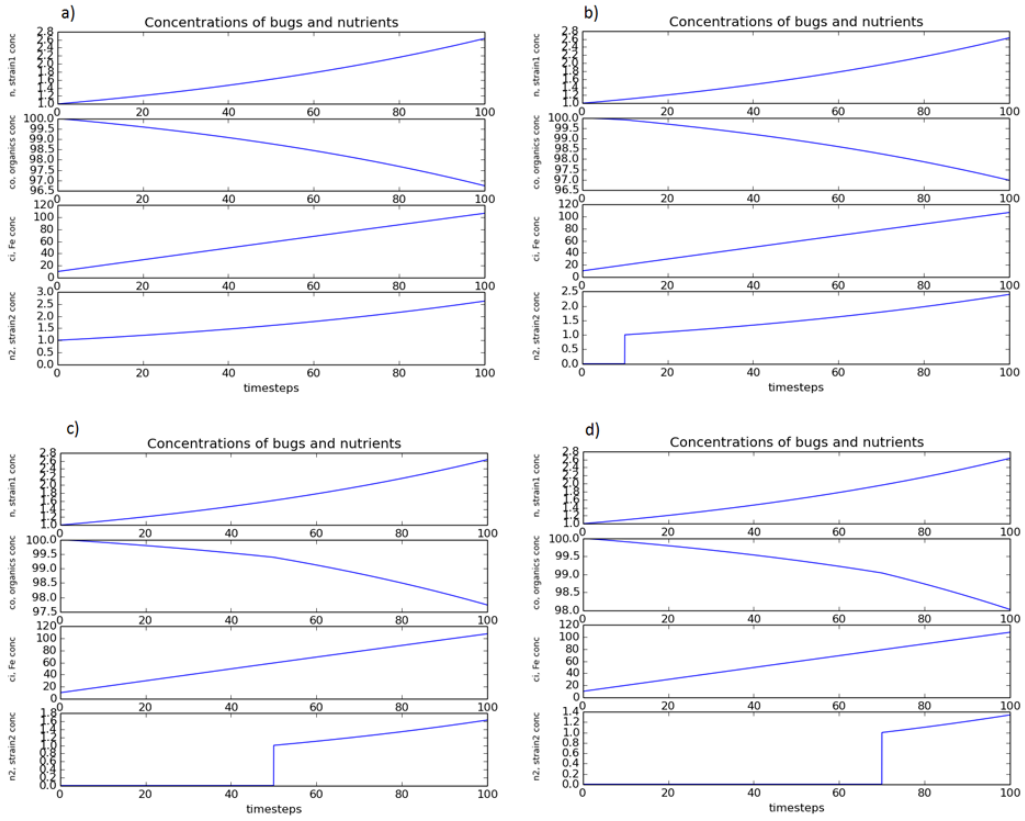


Figure 5.17 *Behaviour of the isolates model with constant input of Fe, but where organics is not re-supplied. The second strain is added after a) not added, b) 1000, c) 5000 and d) 7000. From top to bottom the panels show: n1, the number of organisms of type 1; co, concentration of organics; ci, concentration of Fe; n2 number of organisms of type 2.*

constant that determines the rate of supply of Fe.

The trial runs of the model show that these parameters can be used to model microbial interactions where priority effects are thought to play a role. If all growth parameters are equal between the strains, priority effects will play a role, as a strain that arrives earlier will be able to grow to a higher final concentration as it will have been in the system for longer, and also has potential of utilising the available nutrients unimpeded. It is possible to alter the parameters depending on the strains under study such that priority effects are unimportant, if one strain has a clear growth advantage in terms of significantly higher growth rates or tolerances to the present environmental conditions. Equally, if the time of priority, i.e. the time between when the different strains are added, is short compared to the length of the experiment or the growth rates of the organisms, then priority effects may have a smaller effect on the final community composition.

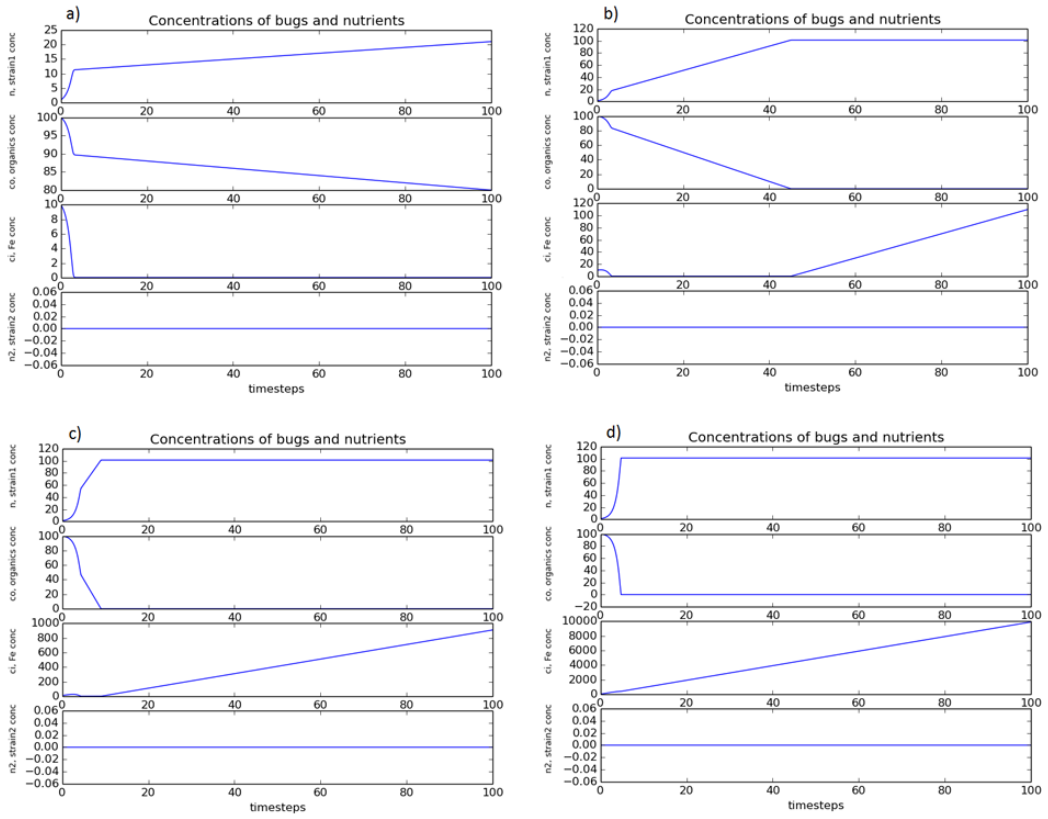


Figure 5.18 *Behaviour of the isolates model with varying input rate of Fe (r), but where organics is not re-supplied. The various values of r are a) 0.1, b) 2, c) 10 and d) 100. From top to bottom the panels show: n1, the number of organisms of type 1; co, concentration of organics; ci, concentration of Fe; n2 number of organisms of type 2.*

If the experimental part of this study had yielded conditions where it would have been possible to accurately measure growth rate and other relevant growth parameters that are used in the model, these would have been entered into the model, and the results would have been compared to experimental data of mixing the isolates together. Thus, it would have been possible to investigate whether the results from the experiment could be replicated using these growth parameters. If there had been discrepancies, it is likely that the interaction and final growth of both strains measured in the mixing experiments was due to parameters that had not been accounted for in the model. As the experimental part of this study did not work out as planned, it was not possible to enter the experimental results in the model, or attempt to draw parallels between the two ways of approaching the problem. The results outlined here show that if the problems encountered here can be overcome, it is a promising dual approach that has the potential to

elucidate the factors involved in creating priority effects in community assembly.

5.5 Discussion

The results from these experiments are varied and provide some useful insights. In addition, some avenues for future investigation are close at hand, as well as some factors of concern in order to design successful experiments.

5.5.1 Are priority effects strong enough to have a significant effect on the survival of two different species mixed together at different times?

The results here both support and challenge the main hypothesis presented, that the first coloniser would have an advantage over the later arrival, and hence grow to higher cell numbers in the final culture. In the first and second experiment conducted here, on strains iso08 yellow and iso13 clear, it is found that the first coloniser grows to higher final numbers, regardless of which strain is added first. In the third experiment, however, where different strains are used, it is found that one strain grows better regardless of whether it was added first or last, in both the combinations of yellow-orange (where yellow dominates) and red-orange (where orange dominates). This means that there is evidence both supporting and challenging the hypothesis.

These results are not necessarily contradictory, as it may depend on the growth rates and restrictions of each organism. The results make sense if the growth rates of the two organisms are comparable, such that priority would be a strong enough variable to create a different outcome on the community depending on the order of colonisation. In an idealised scenario where the initial conditions are equally favourable for both organisms, and they have the same growth rates, priority would play a role on the final community, all other things being equal, as seen in for instance Figure 5.13. If however the growth rates or growth requirements are very different for two species, or the time between adding the strains is short, priority effects can be masked by these other differences, such that one strain is always more dominant in the final community.

It appears that both these scenarios are observed in this study. In the two initial

tests with the iso08 yellow and iso13 white strains, a priority effect is observed, whereby the earliest colonist ends up dominating the end-point community. From the growth curve measurements conducted (Figure 5.12), we see that their growth rates are fairly similar, which could mean that all other things being equal, priority effects would play a role in shaping this community. This ties in with the metacommunity concept, where under similar growth conditions the final community is considered to be shaped by stochastic and dispersal factors [165].

Conversely, in the third mixing experiment, where iso08 yellow was mixed with iso04 orange, and iso01 red was mixed with iso04 orange, the same strain dominates the final communities regardless of whether it was the primary or secondary colonist. In these cases, it is likely that the most successful strain is one that is either best adapted to the conditions or has a faster growth rate, and can therefore out-compete the other strain and grow to greater numbers. Figure 5.12 shows that of the pairs tested in the third mixing experiment, the yellow strain has a higher growth rate than the orange strain, and ends up dominating the community regardless of whether it is added first or last, as is seen in Figure 5.6. Similarly, the orange strain has a higher growth rate than the red strain (final OD readings 0.005 for iso01 red and 0.015 for iso04 orange), and ends up dominating the co-culture both when it is added first and it is added second, as seen in Figure 5.7.

The results observed here complement previous studies looking at priority effects using a select number of isolates. In a study of priority effects utilising yeasts inhabiting different types of nectar, it was found that priority effects played a role, and that the direction of priority was negative, i.e. that early arrivals negatively affected the growth of the secondary colonists [251]. In that experiment, the four yeasts were added in a pairwise sequential manner to PCR tube microcosms with nectar of varying harshness. The experiment was conducted over five days, with initial inoculation of one species into the environment at day one, with the next arrival added after 48h, which is similar experimental set-up and time scales as is used here. The priority effects were seen to vary in strength depending on the types of species used, which also appears to be the case in the experiments presented in this study, and the harshness of the environment. The negative effects of some species on the growth of others by altering the environment have also been successfully modelled [272], supporting the effects observed in this study.

The evidence for priority effects having a role to play in community formation is observed somewhat also in the colonisation experiment that forms a part of

this body of work (Chapter 3). Priority effects appear to be somewhat significant but mostly obscured by other factor in the larger, more natural colonisation experiment. The fact that there is a large homogeneity observed between the colonisation communities suggest that priority effects are not dominant - if they were, it is possible that the end-point communities would have been vastly different. Priority effects may be responsible for some of the differences seen between triplicate identical samples. It is likely that priority effects can be more strongly observed in a smaller community with fewer variables, such as this study here, whereas in the more natural colonisation experiment more factors other than priority affected the final community composition.

The results presented here are a first iteration of how one may begin to answer the question about the importance of priority effects using environmental isolates, but the results must be treated with some caution as there was a large variability observed and not all results were statistically significant, meaning that more replicates, an amended experimental approach, and use of isolates that do not clump are necessary in order to answer these questions fully.

5.5.2 Which growth factors are important for determining whether priority effects play a role?

Due to the problems with clumping experienced in this project, it is difficult to answer this question convincingly. The aim here was to look at three factors: the concentration of Fe as a proxy for inorganic nutrient availability, the concentration of organics, and pH. All organisms have different ranges of these variables within which growth is achieved, so these are pertinent values to consider. These variables were also chosen as they are relatively easy to control and measure in the lab, ensuring that the experiment could remain well constrained. The future directions laid out in the next section outlines some ways in which this question could be addressed with a successful set-up.

In general, the model designed works well for its purpose, and it would be possible to investigate this question further, if the experimental data had been possible to attain. The model allows environmentally-measured parameters to be entered into the system, to test whether specific variables under consideration affect the importance of priority effects in community assembly. If it was possible to measure experimental values of growth rates and growth parameters for isolated

strains of bacteria of interest, these could be entered into the model to see whether experimental results could be replicated, and hence be able to evaluate the effect of various growth factors on priority in community assembly.

5.5.3 Natural variability in growth among triplicate samples for environmental isolates

Relating this data to other experiments undertaken on community assembly in this body of work, this variability in growth rates, as measured by CFU counts on agar plates, may be characteristic of environmental samples. The fact that all isolates tested here indicated some level of variability in growth rates among triplicates and showed visible clumping when growing in liquid culture, suggests that this may be a common phenomenon among strains isolated from an atmospheric inoculum. The experiment shows that priority effects can occur, but the variation is high and often results in a statistically insignificant difference although averages remain different. When considering a full, complex natural community, these effects could be responsible for some of the variation in community composition seen in identical samples in for instance Chapter 3. If the variation in growth seen in this experiment is typical for growth of environmental isolates in a laboratory setting under constrained conditions, the effect is likely to be even larger in a complex natural community, which could explain why communities can diverge under natural conditions as a result of stochastic differences in growth.

5.6 Limitations

5.6.1 Problems with clumping

Significant problems were encountered in this work due to bacterial clumping or biofilm formation. This was manifested in two ways: observable clumps of biomass in the tubes used for growing liquid cultures, and in the spread or variability of counts of CFUs on agar plates, with little homogeneity visible within triplicates, as seen in Figures 5.9 and 5.8. The problem appeared to be that the cells were clumping together in the liquid, growing mostly as one big mass, whereas in an ideal case, cells would be homogeneously distributed

throughout the volume of liquid. When the liquid was swirled around in the tube, the cells would move as one big mass. This was observed to be the case for all environmental isolates used in this study. Some would clump more or less than others, but all strains showed significant amounts of clumping.

The clumping created a problem in this work as it made the CFU counts heterogeneous and unreliable, as there was a large difference between triplicates. It is also possible that the clumping would interfere with the hypothesis testing, if the strains were for instance growing in two separate masses. If the strains were growing spatially separated from each other, and other conditions were fulfilled, such that there were enough nutrients available in the liquid medium and no strain altered the chemical environment significantly, it is possible that no interaction between the strains would be observed, and thus priority effects would not be measured, with the caveat that a similar scenario could occur even if the cells were homogeneously distributed in the liquid. On the other hand, if either or both of the strains were altering the chemical environment, or if nutrients were becoming limited, priority effects may still be present with clumping taking place, although this would be difficult to measure using CFU counts.

It was possible to somewhat homogenise the cultures before plating, by shaking and swirling the tube, and also using a pipette to vigorously break apart the cell clump. This meant that it was possible to take out aliquots of liquid containing cells from the culture, but once these were plated onto an agar plate, it was clear that the liquid was not properly homogenised, as CFU counts varied widely between different triplicate aliquots from the same culture (Figures 5.9 and 5.8). Some variation is always expected, but the variance apparent here was much higher than normal. The clumping was most evident within triplicate samples, but also during dilution series, where a ten-fold dilution of liquid did not result in a ten-fold decrease in CFUs on the agar plates (Figures 5.9 and 5.8).

Many iterations of this experiment were trialled as part of the initial pilot and set-up, using different isolates, isolate combinations and media, which are not presented here; the data presented here are from the experiments which did not experience too much clumping. Some of the problems with clumping can be seen in the dilution series data presented in the third mixing experiment. Repeated attempts were made to homogenise the cultures, and some improvement was seen, but not enough to carry on with the experiment, where reliable cell counts would be crucial for testing the hypotheses and drawing valid conclusions.

The reasons for why cells were growing in clumps are likely to do with stress. This type of growth is commonly seen as a stress response, where EPS (extracellular polysaccharides) is produced to keep the cells together [127]. It is probable that the isolates used in this study were stressed, as they were grown in a minimal medium, which can be considered a harsh environment. The isolates have come from relatively nutrient-poor conditions as they were isolated from environmental rock microcosms, but as they were initially isolated on yeast extract agar as a medium, these may be organisms more used to growing in nutrient-rich environments. It was deemed in this study to use a defined medium, but it may have been possible to design a defined medium that was slightly richer in nutrients, but ideally also possible to be easily altered to growth-limiting or near growth-limiting conditions for the strains involved. Equally, it may not necessarily be the organics limitation that causes the stress response, but it could in principle be anything about the environment which creates this behaviour, including the abundance (or lack of) of other bioessential nutrients such as Fe, N, P etc., or even other variables such as temperature.

It is perhaps not surprising that environmental isolates exhibit unusual behaviour, such as a stress response, when cultured in the laboratory under very different conditions to the situation in the wild. One of the particular objectives of this study was to work with environmental isolates that had originated in environments of interest to this body of work, in order to draw realistic and relevant conclusions about the real environments. When these strains all exhibited problems with clumping, the rest of the experiment was not realised. One option could have been to instead work with model organisms whose behaviour and culturing conditions are well-known, however, this was decided against as it did not fulfil the original objectives of the study. Alternatively, it may have been possible to find a different growth medium, where the isolates under study would not clump in liquid culture, however, the selected medium would need to contain only defined ingredients so that the environments could be replicated and altered exactly as was necessary to carry out these experiments.

The prevalent clumping of environmental isolates under laboratory conditions is an important finding and requires further investigation. In this study, the proportion of isolates that were found to clump was nearly 100%, suggesting that this may be a very common response when sampling environmental strains and subjecting them to an unfamiliar artificial environment. Clumping can make priority effects irrelevant if strains grow as a homogeneous mass rather

than dispersed throughout the liquid, if this means that the strains grow completely spatially separated and without interaction, if neither strain alters the environment and the nutrients do not become depleted. The experimental set-up here cannot assess whether clumping is also prevalent in the environment, or whether it is an artifact of stress related to a new environment. If clumping is a common phenomenon also in the environment, it is possible that priority effects may matter less if species grow in spatially separated locations. The prevalence and causes of clumping require further investigation, with experiments designed to specifically study this phenomenon.

This study highlights some of the difficulties that may be encountered when working with environmental isolates as opposed to model organisms. Currently, much work is done in the laboratory on organisms that are well-known and are known to be relatively easy to work with. The difficulties encountered here show that further effort is necessary in order to be able to cultivate and utilise environmental organisms in the laboratory in order to ultimately be able to test the hypothesis. Model organisms are often not representative of the majority of wild-type strains, and hence more work is needed to understand the best approaches to working with environmental isolates, and develop systematic methods to find suitable methods and conditions to work with each new isolate. As such, garnering more information on environmental isolates and their growth requirements is central. Finding more environmental isolates that are useful in the laboratory would also increase the scope of model organisms available for future scientific endeavours, but the point being made here is that it is also necessary to establish methods by which one can with relative ease begin working with new isolates that are relevant to the environment and phenomenon in question.

5.6.2 Using cell counts to estimate growth

The evidence from this experiment suggests that cell counting is not the best option for estimating growth and population size in the case where cells are not homogeneously distributed in the liquid culture. There are, however, other options of measuring biomass. The approach here was to use visual inspection to distinguish between colonies growing on an agar plates, using isolate strains of different colour to tell them apart in a mixed culture. Thus, methods such as optical density spectroscopy, microscopy, dry weight measurements, assays, or DNA extraction and qPCR (quantitative polymerase chain reaction) would not

offer an easy way of distinguishing the biomass of each strain, although the total biomass of the sample could be established. As this experiment was designed to use simple and quick methods, these other techniques were not considered further, although for future work it is possible that some techniques beyond CFU counts could be modified and calibrated to be used in priority effect experiments.

5.7 Future directions

There is considerable potential to carry out further experiments based on the ideas and experiences from this study. Firstly, the task is to find a suitable set of isolates that grow well in liquid culture, i.e. growing in an even suspension without clumping. In order to achieve this, many strains may need to be isolated and tested under various growth conditions. Depending on the exact rationale, it may be possible to go down the route of using model organisms, or at least organisms that are well known and understood under laboratory conditions. Otherwise one could focus on collecting environmental isolates from the conditions of interest, and isolate these straight onto plates of the selected medium, for the subsequent purification and liquid culturing. With time and effort it will be possible to find isolates that will grow favourably under the laboratory conditions necessary to carry out the experiment, such that growth can be accurately measured, either by plating or OD.

It is necessary to find organisms of different colours, such that their growth can be distinguished on agar plates. Optical density measurements may only be useful to understand the growth of each organism on their own, but not suitable for co-cultures. That being said, one avenue for exploration would be to investigate whether OD readings are possible in a co-culture at different wavelengths for different coloured isolates, hence enabling determination of the growth of each species even in a mixed culture.

The medium used here, M9 supplemented with Fe, may not be ideal, as evidenced by the clumping observed, which may be a stress response, so other defined minimal media may need to be trialled. A more undefined medium may be used, such as yeast extract agar, but it comes at the price of not being able to accurately control the concentration of various nutrients, which has implications for how well the experiments could be replicated in the model, as some parameters may be less well defined. It may still be desirable to keep the medium defined however, in

order to ensure a well-constrained system with a high amount of reproducibility. In that case, one could look at defined media with a richer nutrient content, such that the environmental isolates do not experience stress, and hence clumping.

Once these initial major hurdles have been overcome, more specific mixing experiments can be performed. The experiments will consist of various permutations of adding strains in different orders and combinations, and varying the time elapsed between strains. It is sensible to start with mixing the isolates in pairs, before more strains can be added to the same system. It would be crucial to work with organisms of different colours, such that their growth can be separated and accurately enumerated on agar plates. The first experiment could consist of adding two strains to a flask in pairs with a week's difference, such that one strain has a week to grow in liquid medium before another organism is added. This will be performed for the different combinations of pairs, including reversing the order, and the final community composition will be determined by plating, or if possible, OD measurements at different wavelengths.

Once a working system is set up, the experiment can be refined. Once growth rates and characteristics are well known, it would be possible to ensure that each strain is added at the same cell concentration at the start of the experiment. It would be interesting to study the effects of growth rates or growth requirements on the system, as we hypothesise that priority effects may be swamped by other factors if the organisms have very different growth characteristics. More strains can be added, working with four or eight isolates at once, although the colour and morphology scope may not allow more than a handful of strains to be distinguishable in the same experiment.

It would also be interesting to study the system with rock powder added to the growth medium, such that the experiment can be replicated in a rocky environment. This would ensure a system that is closely related to the real conditions which these organisms have been isolated from, and give results that can lead to real predictions about the natural system. This needs to be approached with some caution however, as making agar plates with rock powder can sometimes be difficult, primarily because rock powder can drive down the pH of the solution, making it impossible for the agar to set, as was found in another experiment that forms a part of this body of work (see Chapter 6). That being said, there are examples of rock powder plate being created successfully, and as mentioned in Chapter 6, it was achieved here under some conditions.

Depending on the success of setting up this experiment, and the conclusions drawn from the initial experiments, further tests can be elaborated and carried out to continue to address the question of how the order of colonisation affects community composition.

The mathematical model presented here was intended to be further developed according to the progress and direction of the isolates experiment, with the aim of being able to characterise the system in the laboratory and determine what factors of growth affect community assembly. The model takes into account variations in organics and Fe, which is considered the main limiting nutrient, and models the interaction between two different strains, which can have different growth characteristics. The model could be further extended to include dependencies on several nutrients. For a more complete picture it should include a more detailed dependence on pH, as it is likely that the different strains have different pH tolerance. Initially, pH tolerance can be modelled as a step function, with zero growth outside some range, and maximum growth inside the range. It is also possible to use a trapezoid shape or similar to describe growth as a function of pH. A dependence on temperature could also be implemented, again with a step function or trapezoid appearance. The model can then be extended to include as many strains as are used in the isolates experiment. The model would thereafter be guided by the experiment, and experimental data will be used as input in order to mimic the laboratory situation as closely as possible.

5.8 Conclusions

In conclusion, this experiment has yielded further insight into the question of whether priority effects influence the resultant community in microbial community assembly. The results from this study indicates that priority effects may play a role in the assembly of systems when growth rates of various organisms are well matched, but may be less important when growth rates or other characteristics are very different between organisms.

The study highlights some of the issues experienced when working with environmental isolates, and as such, indicates that more work is needed to work on real organisms in natural systems, as opposed to model organisms which may not be representative of the behaviour of the majority of organisms in nature. Thus, this experiment gives some useful insights while also indicating some future avenues

of scientific exploration.

Chapter 6

The role of the microbial community in affecting environmental alteration

The longevity and persistence of a complex microbial community is affected by how the microorganisms themselves alter the environment away from its original state. In rocky environments, environmental alteration is most readily detected or experienced in terms of changes in pH and elemental leaching from the rock substrate. These changes feed back to the resident community, thus constraining the complex community during the process of colonisation and succession. There is still uncertainty about how a soil community is shaped by and affects elemental leaching in different rock substrates. In addition, little work has been conducted on direct comparisons between rock weathering by natural microbial communities under aerobic and anaerobic conditions. An experiment was set up to compare the ability of a soil community to affect elemental leaching on two different igneous rock types under aerobic and anaerobic conditions. It was hypothesised that the microbial community would favourably leach certain elements from the rock compared with the abiotic controls, and that the elements leached would be different between the two rock types. It was also hypothesised that leaching rates would generally be slower under anaerobic conditions compared to aerobic conditions, as anaerobic metabolisms are generally slower and less efficient. It was found that leaching rates were higher under biotic conditions for certain elements, whereas it was lower for others, and that both rock type and the presence or absence of oxygen affected leaching patterns. This experiment supports the idea

that microorganisms alter their environment away from its original state, which has implications for subsequent community assembly and alteration.

6.1 Introduction

Weathering of rock is the first step in soil formation, and the role of microorganisms in weathering is of industrial and agricultural importance. This topic is interlinked with the global carbonate-silicate cycle, as it is not known to what extent microbial rock weathering contributes to this [23]. Understanding the differences in weathering in the presence and absence of oxygen has relevance for interpreting the Earth's history, which has experienced significant anoxic and oxygen-poor periods, as well as different environments today. In this experiment, weathering of granite and gabbro was studied over two months, using a soil community as an inoculum. Elemental leaching was measured by ICP-OES, which showed that the bacterial community enhanced leaching of certain elements. The aim was to study microbial weathering of two different rock types under aerobic and anaerobic conditions, drawing inspiration from key studies such as Uroz et al. [247], Wu et al. [280], Wu et al. [279] and Bennett et al. [21] (Section 2.5), in order to better understand biosphere-geosphere interactions pertaining to microbe-rock interactions.

Microorganisms interact with the geosphere through the weathering of rocks. Broadly speaking, weathering refers to any process whereby rocks are broken down *in situ* and is carried out by mechanical, chemical and biological agents working in synergy. For the purposes of this study, only weathering by chemical or biological means will be considered, which can be described by geochemical alterations of geological material. Weathering is often coupled with erosion, which physically moves weathered rock fragments to new locations. Through the process of weathering, microbes facilitate the release of nutrients which can be taken up by other organisms, such as plants and fungi, making them fundamental agents in many ecosystems [247]. Microbe-mineral interactions can be both detrimental and beneficial to society, with examples of generally problematic microbial weathering is tooth decay and break-down of mineral-based historic monuments and artefacts, whereas biomining and biological fertilisers are counted as useful applications. On larger scales, microbial rock weathering may have important climate implications, through contributions to the carbonate-silicate cycle by the weathering of silicate rocks. Weathering releases cations that deplete

CO_2 levels in the atmosphere [152], with rocks eventually subducted into the Earth's mantle on geological timescales, after which CO_2 is released back in the atmosphere through volcanism. The balance between weathering and volcanism determine the instantaneous concentration of atmospheric CO_2. The weathering of rocks by microorganisms feeds directly into this process, influencing the level of greenhouse gases and therefore affecting climate.

There is an important feedback process between microbial rock weathering and community assembly, phenomena which are both of interest for this work (for the study on community assembly, please see Chapter 3). When microorganisms colonise a new rock substrate, they facilitate elemental release from the rock, either as an active process in order to scavenge nutrients, or as a passive byproduct of their metabolism. This has the effect of altering the geochemical environment away from its original state, which impacts the survival and structure of the rock-dwelling ecosystem as a whole [67]. Primary colonists thus need to be able to utilise nutrients from the rock and atmosphere for their survival, whereas secondary species arrive to a more benign and nutrient-rich environment. These new arrivals may in turn again alter the environment through their presence, and the larger and more versatile the community gets, the greater the potential impact on the rock substrate. Thus, microorganisms that colonise fresh rock surfaces have to acquire some or most of their nutrients from the rock, causing the rock to weather. Weathering in turn alters the abiotic environment, meaning that new species can take up residence, as a new geochemical environment provides nutrients that were previously absent, opening up for new metabolic pathways. This means that new species can take up residence, or in some cases that the resident organisms will alter their metabolism if a new, more favourable pathway is found. These changes, with new species immigrating into the system, or dormant species becoming active, results in a complex community, which continues to weather the rock. For instance, heterotrophic organisms may not be able to take up residence in a rocky, organics-poor environment until it has been colonised by Cyanobacteria that fix carbon from the atmosphere, hence providing organics for the subsequent heterotrophic community. These new heterotrophic organisms may in turn generate organic acids as a byproduct of their metabolism, which will further the weathering process. The continued weathering through microbial metabolism creates a dynamic system whereby nutrients and resources can be recycled through the system, and new metabolisms can serve to release new forms of nutrients, which can further enhance the diversity of the community. With time, the system goes from a simple community to a complex feedback

loop, where community assembly is both a product of and a catalyst for rock weathering. Thus, understanding the global impact of microbial weathering requires knowledge of both community assembly and environmental alteration.

6.2 Unanswered Questions and Hypotheses

6.2.1 Questions to be answered

The work undertaken in this project is based on questions regarding biosphere-geosphere interactions that have not yet been answered.

- To what extent are some parts of the planetary crust more susceptible to microbial weathering? Here, weathering of two rock types, granite and gabbro, is compared, and elemental leaching as well as community composition monitored over seven weeks, using a soil inoculum at the outset. It was hypothesised that there would be noticeable differences in leaching patterns between the two rock types, and that gabbro may overall show a higher amount of leached elements, due to higher concentrations of most major and trace elements present in this rock type. Also, it is hypothesised that the biological systems will favour leaching of certain elements compared to the abiotic controls.

- How does the planetary crust influence the emergent complexity of microbial communities? It was hypothesised that the communities would be broadly similar but distinct, such that at higher taxonomic levels the communities appear similar, but with larger differences at genus or species level. It was also hypothesised that granite would host a community with lower diversity than gabbro, due to slower release rate of nutrients as it has a higher silica content [190]. It was further hypothesised that these would be complex communities with less diversity than a soil community, with no single species dominating the community in terms of the relative abundance of the sequences.

- How would major planetary change, such as the oxidation of the atmosphere, affect microbial weathering? Here, microbial weathering is studied using aerobic and anaerobic microcosms. It was hypothesised that microbial weathering would be slower under anaerobic conditions, as organisms are

likely to grow slower by anaerobic metabolisms, and that the community composition will be different under aerobic and anaerobic conditions [52].

- How do pioneer microbial communities change or enhance habitability for later colonists? Habitability was assessed using elemental leaching as a proxy for the geochemical environment. It was hypothesised that the geochemical composition would be different between one and seven weeks of weathering, and that the pH in the microcosms would have altered.

6.3 Methodology

The experiment consisted of laboratory microcosms with two different types of crushed rock, granite and gabbro (Section 2.6). The microbial community was established from a soil inoculum, collected from an area with igneous bedrock, and was added to the microcosms through a series of transfers in order to minimise the amount of soil in the final analysis. The experiment was repeated under both aerobic and anaerobic conditions, and ran for 7 weeks with fortnightly time points. At each time point, data was collected to monitor environmental changes through pH analysis and elemental release, which was measured through ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometry). At the final time point, data was also collected for 16S rRNA sequencing and cell counts. Some of the initial rock powder was analysed for mineral and elemental composition through XRF (X-ray fluorescence) and XRD (X-ray diffraction). Furthermore, sulfide production in the anaerobic cultures was examined through a sulfide assay, and sulfide precipitates studied using SEM-EDS (Scanning Electron Microscopy Energy-dispersive X-ray spectroscopy).

6.3.1 Sample Collection

It was desirable to use an environmental soil inoculum from areas with granitic and gabbroic bedrock and a search for suitable sample sites was undertaken, with the aim of finding a granitic and a gabbroic area in close proximity to each other, with similar environmental conditions. Given Scotland's varied and rich geology, several possibilities were explored before settling on the Isle of Skye as the optimal site, due to the presence of suitable sampling locations for granite and gabbro soils that were easily accessible by road within 10 miles of each other.

6.3.1.1 Field trip to Isle of Skye

The field trip to Skye took place on January 26-27, 2015. Sampling was conducted both aerobically and anaerobically at every location, with aerobic samples stored in sterile Whirl-Pak bags and anaerobic samples stored in sterile Duran bottles (Figure 6.1). Four sample sites each for gabbroic and granitic soils were chosen. Samples were collected on hillsides facing the ocean within a few hundred metres from the A87 on the southeastern part of the island from environments such as rivers, streams, woodland, heather slopes and erosion banks, to represent a wide variety of soil environments of granitic and gabbroic origin. Aerobic samples were collected from the topsoil at each site, while anaerobic samples were collected rapidly at 30cm depth directly into deoxygenated Duran bottles. Gabbroic samples were collected at the Broadford Gabbro Complex (NG 62072 25604; 57.258996°N, -5.9469097°W), while granitic samples were taken around the banks of Loch Ainort (NG 53462 26433; 57.261873°N, -6.0900936°W) (Figures 6.2 and 6.3, more detail in Appendix C). Samples were kept outside overnight with a low of 3°C, and brought back to the laboratory in Edinburgh where they were stored at 4°C before use.

Figure 6.1 *Aerobic samples in bags, anaerobic samples in bottle*

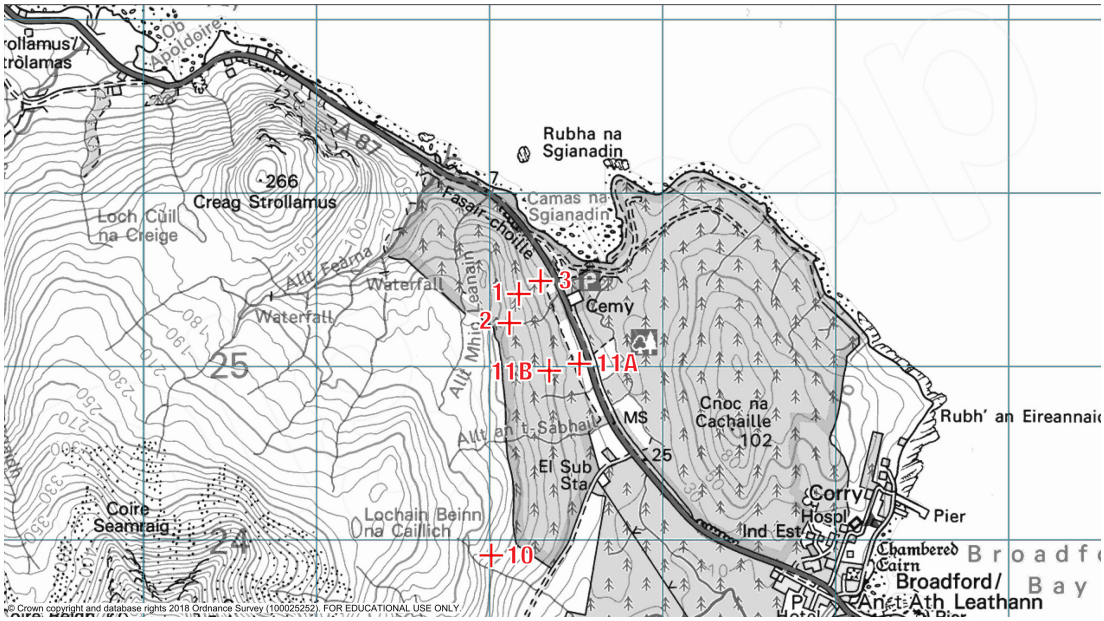


Figure 6.2 *Broadford Gabbro Complex sample sites 1, 2, 3, 10 and 11. One square is 1km across.*

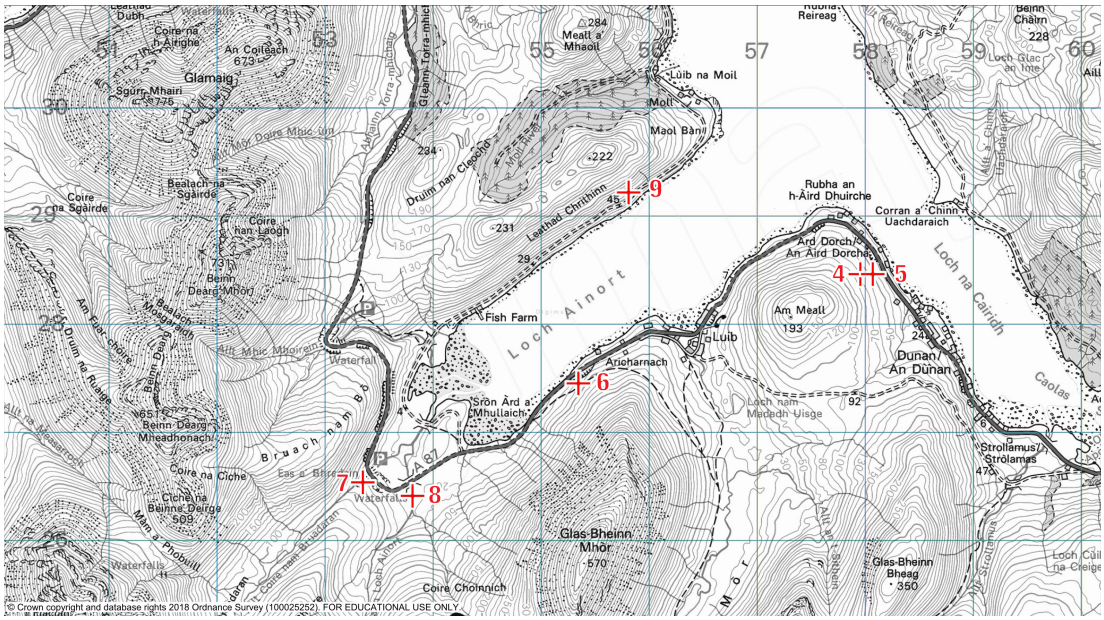


Figure 6.3 *Loch Ainort granite sample sites 4-9. One square is 1km across.*

(a) Gabbro sampling location overview

(b) Granite sampling location overview

(c) Granite sampling location overview

(d) Granite sampling location overview

(e) Gabbro sampling location detail

(f) Gabbro sampling location detail

(g) Granite sampling location detail

(h) Granite sampling location detail

(i) Gabbro sampling location overview

(j) Granite sampling location detail

(k) Granite sampling location detail

(l) Gabbro sampling location detail

Figure 6.4 *Sampling locations for granitic and gabbroic soils on the Isle of Skye, Scotland, January 26-27, 2015*

6.3.2 Soil Inoculum

A mixture of the soil inoculum was created from the soil samples collected on the Isle of Skye. First 10g of samples 1, 2, 3, 6, 7, 8, 9 and 11 were weighed out. These were thoroughly mixed and stored at 4°C until further use. Note that two mixtures were made: one aerobic and one anaerobic, using the aerobic and anaerobic soils respectively from the sample sites listed. Thus, the soil inocula for aerobic and anaerobic conditions used in this experiment were not identical: the soils had been sampled in the same locations but at different depths.

6.3.3 Rock Characterisation

In this project, two types of igneous rocks are utilised: granite and gabbro. The rocks used in this study were bought from commercial suppliers, in order to ensure bulk homogeneity for use in a controlled experiment. Granite and gabbro are intrusive igneous rocks that share some common characteristics, but also have distinct features. These two rock types were chosen as they represent the two major constituents of the Earth's crust, with granite making up the majority of the continental crust, and gabbro being the main component of the oceanic crust. Thus, studying the weathering patterns and capabilities for hosting microbial communities of these two rocks gives as extensive an image as possible of microbial weathering at global scales. It is hypothesised here that granite would host a community with lower diversity than gabbro, due to slower release rate of nutrients [190], [276]. For a further description of the rocks under study, please see Section 2.6.

6.3.4 Rock crushing and preparation

The rocks used in this study were crushed, sieved and sterilised before being placed in the microcosms. Rock crushing was conducted under the supervision of Nick Odling in the School of Geosciences at The University of Edinburgh. The rocks were first cut into smaller pieces before being crushed with a jaw crusher. The resulting pieces were sieved to a size range of 63-1000μm, with some additional crushing taking place by hand using a steel pestle and mortar.

6.3.4.1 Rock sterilisation

The crushed rock samples were sterilised by furnacing. The rocks were distributed into foil parcels of 10g each that were left slightly open in order to let moisture escape. The samples were first heated at 90°C for 2h in order to drive off water, after which the foil parcels were sealed and the samples heated at 500°C for 4h in order to drive off any organics and ensure that the samples became completely sterilised. When setting up the experiment, one foil parcel of 10g was used per microcosm, with microcosms with added rock being autoclaved before inoculation, thus ensuring that the rocks were definitely sterile at the onset of the experiment.

6.3.5 Mineral analysis of rocks under study - XRF and XRD

The crushed rock samples were analysed by X-ray diffraction (XRD) and X-ray fluorescence (XRF) in order to determine their mineralogical and elemental composition, respectively. Analysis was carried out with the help of Nick Odling in the School of Geosciences, The University of Edinburgh. Firstly, the samples were crushed down to a fine powder, using a pestle and mortar, which was then mounted onto discs that were used to hold the samples in place during analysis. XRD was conducted using a Bruker D8 Advance with Sol-X Energy Dispersive dectector and XRF was conducted using a Panalytical PW2404 wavelength-dispersive sequential X-ray spectrometer. XRF analysis was carried out for both major and trace elements.

6.3.6 Growth Medium Selection

The collected soil samples enabled the setting up of an initial weathering enrichment in flasks containing sterile rock samples, mixed soil inoculum and medium. For this experiment, it was desirable to devise a nutrient-poor medium containing the minimum requirements of essential elements, where a community of microbes would have to attain most of their nutrients from the rock, by utilising suitable proportions and supplying as little as possible of the necessary nutrients. This way, the microbial community would be forced to utilise the rocks to find most of their nutrients, while the absolutely necessary elements would be supplied through the medium. Such a community would thus have a high potential for

rock weathering activity and thus be of interest to the study.

The elements that have to be supplied through the medium are C, N, P and S. C is essential as it makes up about 50% of the dry weight of cells and is a constituent of most proteins, fats, carbohydrates and lipids. N is used in the production of DNA, RNA, amino acids and proteins. P is essential for the formation of nucleic acids and phospholipids. S is a common constituent of amino acids and coenzymes.

The medium was designed to enrich for a variety of metabolisms, by supplying glucose as a carbon source for heterotrops, as well as opening up the possibility for sulfate reduction through $MgSO_4$ and ammonium oxidation using NH_4. From the nutrients in the rocks, other metabolisms would also be possible, such as iron oxidation and others. The essential elements N and P were supplied in the medium so that organisms would not have to fix these directly, as only specialised organisms are able to do so.

It was desired to limit the supply of Ca and Mg, although it is not always be possible as it might be a constituent of compounds supplied as for instance an S source. Too much Ca could allow the microbes to grow independently of the rocks as they might then have all the nutrients they need. Micronutrients, cations, trace elements and inorganics should in general be limited as much as possible. Here, a small amount of Mg was supplied in the medium through $MgSO_4$ as the sulfate source, whereas Ca was not supplied.

It was desirable to choose a suitable carbon source that would be general enough to allow for a wide diversity of organisms, while still being defined to ensure that the initial medium was easy to replicate for subsequent transfers. The two main options considered were glucose and yeast extract. Of the various options for a sugar-based carbon source, glucose was considered as it is a simple sugar that can support a high diversity of species. For instance, many species using complex sugars break these down into simpler sugars first, and can thus also utilise glucose. Glucose has the advantage that it is well defined but the disadvantage is that it restricts growth to those organisms which can use glucose for growth, and excludes those that cannot. Yeast extract is useful as it is natural and consists of dead organic material and can hence be a good analogue for nutrients available on the early Earth. However, yeast extract has the disadvantage that it is not well defined and there can be significant heterogeneity between batches, and the supply of additional nutrients is hence poorly constrained. If yeast

extract is supplied, it has to be in very low concentrations. Constituent nutrients which occur in small quantities in the yeast extract will appear in even smaller concentrations when diluted into the final medium. It was decided to use a small amount of glucose as the C source, as it would enable to medium to be better defined.

The medium selected was a modified minimal weathering glucose-based media adapted from Wu *et al.* [280]. The experiment was set up simultaneously under aerobic and anaerobic conditions, with appropriate modifications for the anerobic medium. In order to select an appropriate medium, the first medium trialled was the one listed by Wu *et al.* [280]. This set-up however did not appear to yield large amounts of growth, and it was hypothesised that the minimal medium might not be rich enough to enable the growth of an environmental community, as the medium had been designed for a single organism with known growth capabilities. Therefore, a second set of aerobic enrichments was set up, with the aim of optimising the medium in order to ensure a microbial community with higher biomass. A total of eight different media were tested, half of which contained a phosphate buffer, with the other half containing the phosphate source without buffering capacity from the paper by Wu *et al.* [280]. The choice to exchange the phosphate source for a phosphate buffer was trialled in order to facilitate transfers by ensuring the pH stayed relatively constant. The transfers had the purpose to reduce the amount of soil in the final stage, but it was necessary to ensure a smooth transition for the community, by keeping environmental variables as similar as possible between the transfer stages. In a short pilot study, both the buffered and unbuffered media were tested for nutrient concentrations of x1, x10, x100 and x1000 of the original medium, however, the x1000 media had some components at x100, as the amounts required for x1000 concentration were so high in some instances that they led to saturation. Using a x100 concentration for some of the components in the x1000 instances was thus regarded as the best option, despite this affecting the ability to compare concentrations directly. Glucose levels were kept constant in all media, and gabbro be used as the rock substrate in the medium selection process. The different concentrations of media can be seen in Figure 6.5. Growth was studied by microscopy and plate counting, revealing higher CFU levels and cell densities in the unbuffered media compared with the buffered equivalent. Nutrient concentrations appeared optimum at x10 of the original, unbuffered medium, which was thus used for subsequent experiments.

The medium used for this experiment can be seen in Table 6.1. After mixing the

Wu et al. media buffered to pH 7.4:		Wu et al. media unbuffered, pH 7.4 at outset:	
x1 concentrations:		x1 concentrations:	
Glucose	0.2 g/L	Glucose	0.2 g/L
KCl	0.0005 g/L	KCl	0.0005 g/L
MgSO4	0.0005 g/L	MgSO4	0.0005 g/L
NH4Cl	0.04 g/L	NH4Cl	0.04 g/L
NaH2PO4•H2O	0.031 g/L	KH2PO4	0.0003 g/L
Na2HPO4 (anhydrous)	0.11 g/L		
X10 concentrations:		x10 concentrations:	
Glucose	0.2 g/L	Glucose	0.2 g/L
KCl	0.005 g/L	KCl	0.005 g/L
MgSO4	0.005 g/L	MgSO4	0.005 g/L
NH4Cl	0.4 g/L	NH4Cl	0.4 g/L
NaH2PO4•H2O	0.31 g/L	KH2PO4	0.003 g/L
Na2HPO4 (anhydrous)	1.1 g/L		
X100 concentrations:		x100 concentrations:	
Glucose	0.2 g/L	Glucose	0.2 g/L
KCl	0.05 g/L	KCl	0.05 g/L
MgSO4	0.05 g/L	MgSO4	0.05 g/L
NH4Cl	4 g/L	NH4Cl	4 g/L
NaH2PO4•H2O	3.1 g/L	KH2PO4	0.03 g/L
Na2HPO4 (anhydrous)	11 g/L		
X1000 concentrations:		x1000 concentrations:	
Glucose	0.2 g/L	Glucose	0.2 g/L
KCl	0.5 g/L	KCl	0.5 g/L
MgSO4	0.5 g/L	MgSO4	0.5 g/L
NH4Cl	4 g/L	NH4Cl	4 g/L
NaH2PO4•H2O	3.1 g/L	KH2PO4	0.3 g/L
Na2HPO4 (anhydrous)	11 g/L		

Figure 6.5 *Different weathering media trialled, both buffered and unbuffered modifications of Wu et al. [280] at concentrations x1, x10, x100 and x1000 (note that x1000 has x100 concentration for some components.)*

reagents together, the medium was pH balanced to pH 7.4 using NH_4OH if the pH was too low, and HCl if the pH was too high. Note that the pH only needed a small amount of adjustment, so very small quantities of NH_4OH and HCl were needed. At this point, each time the medium was made up, it was split into two equal aliquots, one for aerobic and one for anaerobic medium. The aerobic part was set aside as it was ready to be autoclaved, while the anaerobic medium needed further treatment.

The anaerobic medium was treated with resazurin (indicator) from 0.1% stock solution in fridge, using 1ml for 1l media, giving a final concentration of 0.001g/L in the medium. The dye has a blue colour before autoclaving, after which it goes clear. It serves as an indicator of the presence of oxygen in the anaerobic microcosms, as the dye turns irreversibly pink at the formation of resorufin in the presence of aerobic respiration. The medium was then bubbled for 30mins

using N_2 gas, by using two needles through the bung, one through which gas was entered, and one which allows excess oxygen to escape. The needle for the gas had a plastic tube with small holes at the bottom attached to it, ensuring that the gas was bubbled through the whole medium. After 30mins, the loose needle was removed, and the needle with the tube removed immediately after (the tube just falls into the bottom of the bottle and stays there without interfering with the experiment). Finally, the reducing agent cysteine was added to the medium, by weighing out 0.5g/L powder L-Cysteine HCl in sterile Eppendorf tube. A 1ml aliquot was removed from the medium bottle using a needle and syringe and mixed with the cysteine powder in the Eppendorf cup, before the mixture was placed back into the medium using the syringe and needle. After this, the aerobic and anaerobic media were sterilised by autoclaving at 121°C for 20mins.

Table 6.1 *Weathering medium at x10 concentration, based on Wu et al. [280].*

Reagent	Amount
Glucose	0.2 g/L
KCl	0.005 g/L
$MgSO_4$	0.005 g/L
NH_4Cl	0.4 g/L
KH_2PO_4	0.003 g/L

6.3.7 Liquid-rock ratio

When studying weathering, it is important to get the liquid-rock ration right such that it is possible to observe the effects of weathering. Wu *et al.* (2007, 2008) [279], [280] use a ratio of about 4g rock/400ml liquid for their experiments. Here, a slightly higher rock-liquid ratio was desired, so that enough leaching would take place in order to be able to measure it over the short timescales, 7 weeks, involved. Hence, this experiment was set up with 10g rock per 50ml liquid (5:1 liquid:rock ratio).

6.3.8 Pilot study

A pilot study was conducted, in order to trial the set-up and try out different media. Both aerobic and anaerobic microcosms were set up, using sterilised rocks, a soil inoculum and growth medium.

Aerobic microcosms were set up in polycarbonate Nalgene flasks of 250ml (Nalgene, Rochester, NY, USA) and sealed with foam bungs and tinfoil, whereas the anaerobic microcosms were set up in glass 100ml serum bottles with blue bytul rubber stoppers and sealed with an aluminium seal crimper. Bottles were sterilised by autoclaving before the experiment was set up.

A mixture of the soil inoculum was created from the soil samples collected on the Isle of Skye. First 10g of samples 1, 2, 3, 6, 7, 8, 9 and 11 were weighed out. Two mixtures were made: one aerobic and one anaerobic, using the aerobic and anaerobic soils from the sample sites listed.

Into each sample bottle was put 10g of sterlised rock and 2g of mixed soil inoculum. After this, 50ml of medium was added to each bottle, and the bottles sealed. Each condition (granite aerobic, granite anaerobic, gabbro aerobic and gabbro anaerobic) was set up in triplicate. Negative controls were set up with rock and medium, but without the soil inoculum. The microcosms were stored on the bench at ambient temperature of 21°C.

After one week growth was estimated using fluorescence microscopy by staining with SYBR Gold DNA binding dye (Life Technologies, UK). An aliquot of 200μl was removed from each sample in a sterile manner. Staining was done from a x20 working stock solution of SYBR Gold by adding 10μl of stain to 200μl of sample liquid. Samples were incubated in the dark for 15mins before being imaged. The cells were imaged using a Leica DM4000B microscope (Leica Camera AG) at x100 magnification under blue light from fluorescence prism I3. Counting was conducted in order to get a general understanding of the growth taking place.

Initial microscopy and plate counts showed some growth for all conditions, but no great density of organisms (Figure 6.6). This meant that an attempt was made to find a more suitable, modified growth medium, as outlined in Section 6.3.6. Having thus optimised the growth medium, new enrichments were set up, which were used as the basis for subsequent transfers and weathering experiment.

6.3.9 Setting up the experiment

After the initial trials regarding the experimental design and methodology, the actual experiment was set up, and three transfers were conducted before measurements took place. The experiment was set up using the methodology as

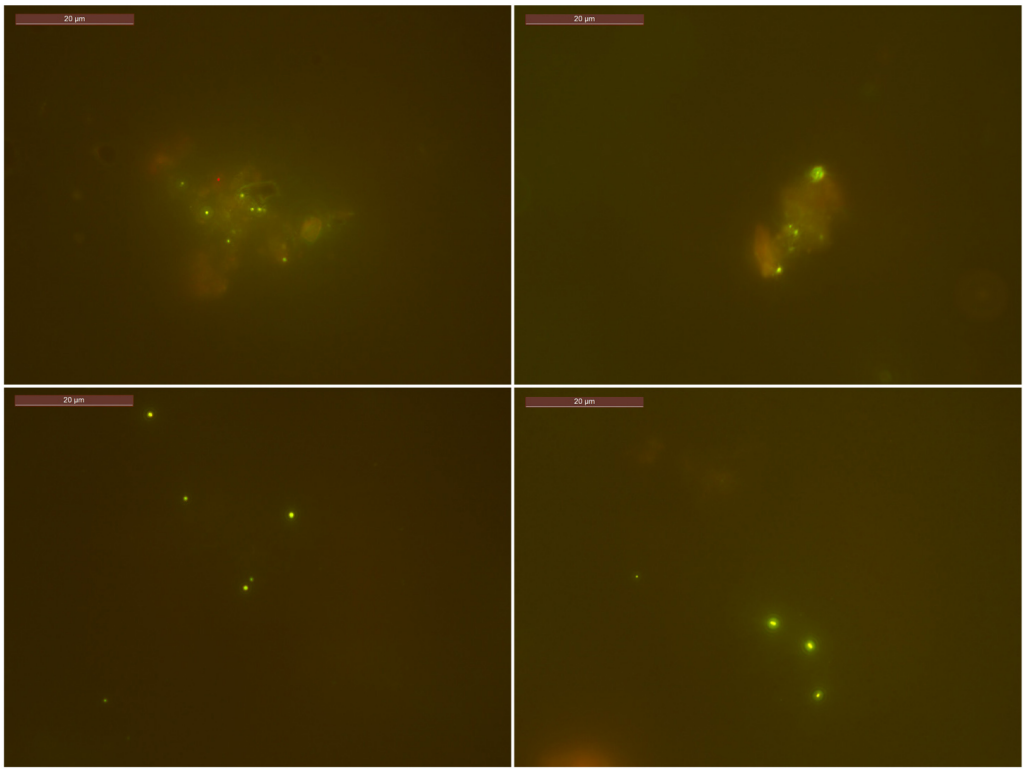


Figure 6.6 *Fluorescence microscopy during pilot study, where different weathering media were trialled, both buffered and unbuffered modifications of Wu et al. [280] at concentrations x1, x10, x100 and x1000. Scale bars are 20µm.*

detailed above according to four conditions in triplicate: gabbro aerobic, gabbro anaerobic, granite aerobic and granite anaerobic. Each condition was set up together with a set of triplicate negative controls for the final set-up, but only one control per condition for each stage of the transfer. The aerobic samples were set up in a laminar flow hood, whereas the anaerobic samples were set up in an anaerobic chamber. To each flask was added 10g of furnaced, crushed rock of either rock type, after which the flasks were sealed with foil and autoclaved. After the flasks and substrate had been properly sterilised, 2g of mixed soil inoculum was added to each flask. Note that the aerobic and anaerobic soil inocula were different mixes, using the respective aerobic or anaerobic samples from each sample site used. Once the soil had been added, the final step was to add 50ml of medium (aerobic or anaerobic as appropriate) to each flask. The aerobic samples were sealed with a foam bung and tinfoil, whereas the anaerobic samples were sealed with a blue butyl rubber bung and crimped with an aluminium seal. A gas exchange was performed on the anaerobic samples, which were bubbled for 2mins with a $N_2/CO_2/H_2$ (85/10/5 %) mix (the mixture in the anaerobic chamber was N_2/H_2 (95/5%)). The samples were stored on the bench at a temperature of

21°C. Growth was monitored by aseptically removing 200μl aliquots and imaging them using fluorescence microscopy for cell counts. The typical density of cells after one week can be seen in Figure 6.7. Aerobic sampling was carried out in a sterile flow hood by removing the foam bung and using a sterile pipette to withdraw liquid and a sterile spatula to take out rocks. Anaerobic sampling was carried out in a anaerobic chamber by removing the foam bung and using a sterile pipette to withdraw liquid and a sterile spatula to take out rocks. The amount of rock was in both cases measured directly into a sterile Eppendorf tube placed on a set of scales to ensure that the right amount was taken out.

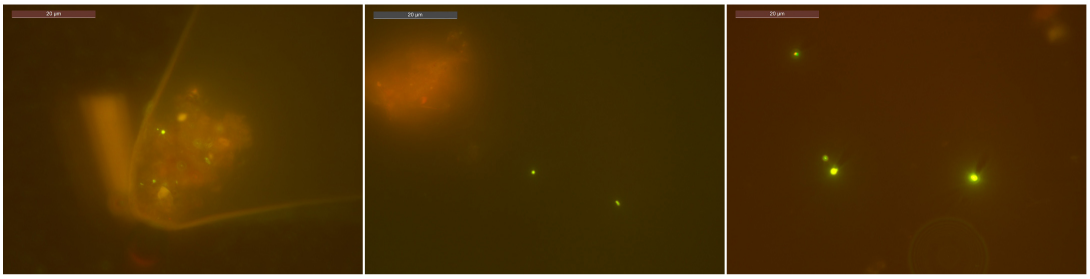


Figure 6.7 *Fluorescence microscopy one week after the experiment was set up. Scale bars are 20μm.*

6.3.10 Transfers

Three transfers were conducted before the experiment began, in order to have a minimal amount of soil carried over into the final microcosms. Each transfer was conducted after 48 days, which was found to be long enough to develop a complex community with noticeable diversity. In order to establish the time frame, the diversity before the first transfer was assessed by performing a DNA extraction, polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE), as outlined in Section 6.3.11. The various stages of setting up and transferring are detailed below, with a graphic displayed in Figure 6.8:

- Set-up - 1 May 2015
- First transfer - 18 June 2015
- Second transfer - 5 August 2015
- Final transfer - 22 September 2015

For each transfer, new sterile microcosms were created as described in Section 6.3.9, by adding 10g of crushed rock and 50ml of sterile aerobic or anaerobic medium as appropriate to aerobic and anaerobic bottles. For the controls, this was the final set-up, and no transfer was conducted, but merely new microcosms set up at each transfer point. To the actual sample microcosms, a transfer of both soil and liquid was conducted. Firstly, the old microcosm was mixed well by shaking, and then 1ml of liquid transferred to the new microcosm. The anaerobic microcosms were opened up in the anaerobic chamber such that it would be possible to scoop out some of the rock. After transferring the liquid, 1g of rock was scooped out of the old microcosm and placed into the new microcosm. In order to ensure that 1g of rock was transferred, the correct amount of rock was weighed out into a sterile 1.5ml Eppendorf tube using a set of scales, before being added to the new bottles. The new microcosms were sealed, and in the case of the anaerobic microcosms, bubbled, and stored as described in Section 6.3.9.

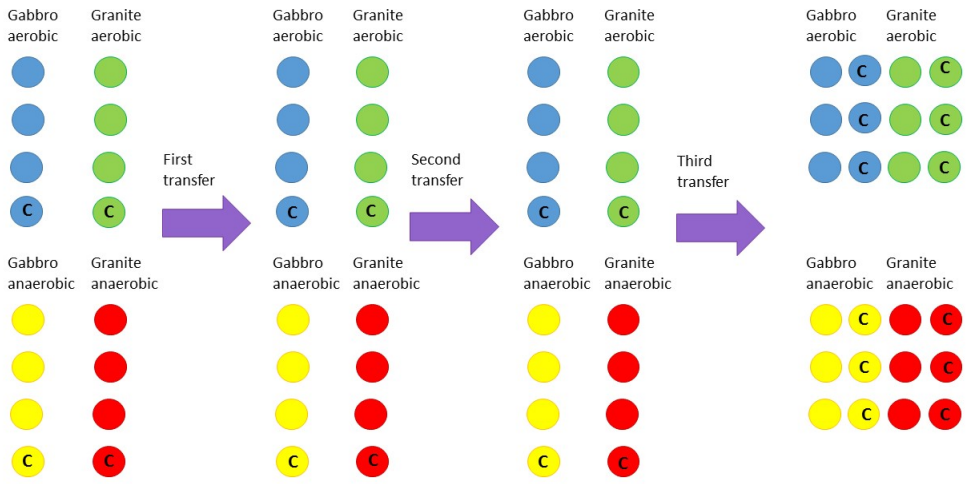


Figure 6.8 *Illustration of the transfers made in the weathering experiment before the final samples were set up for taking measurements for analysis. C=control. Note that only one control per condition was used until the final set-up when the measurements were taking place, where triplicate controls were used.*

6.3.10.1 Microscopy and plating during transfer period

In order to monitor the growth of the samples during the course of the transfers, cell counts were conducted using microscopy. Some plating on 0.2% yeast extract agar (2g/L yeast extract (Oxoid, UK), 20g/L agar bacteriological no.1 (Oxoid,

UK)) plates was also conducted, in order to assess viability of the samples. Microscopy was conducted as described above in Section 6.3.8. The typical density one week after the first transfer can be seen in Figure 6.9.

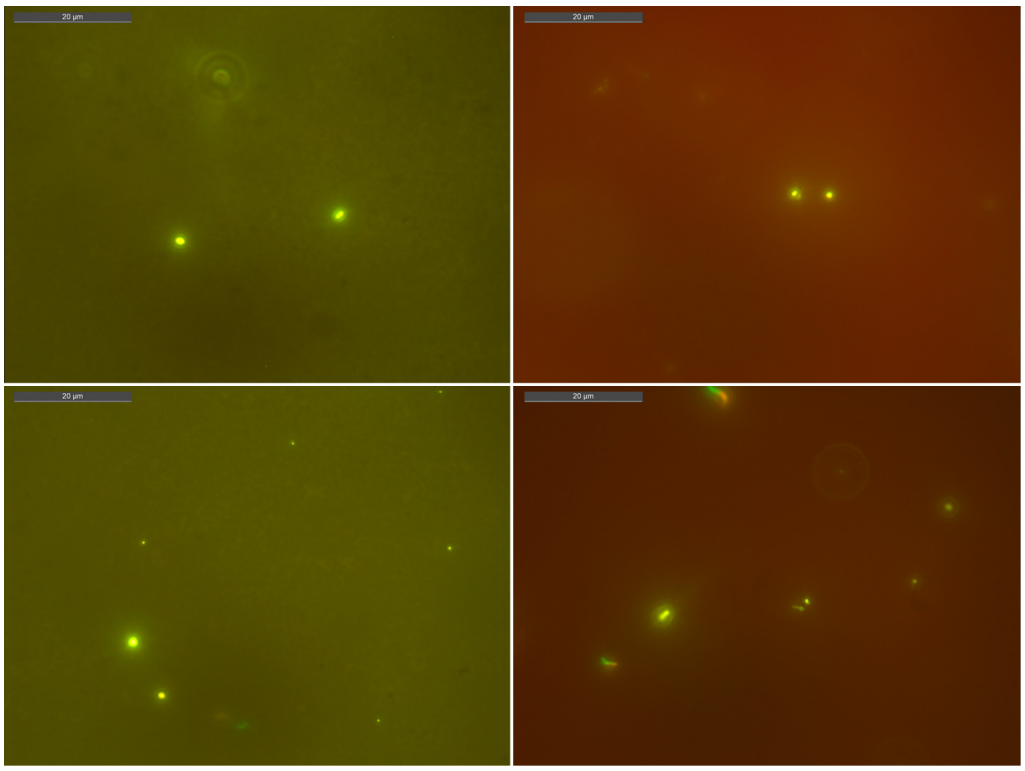


Figure 6.9 *Fluorescence microscopy one week after the first transfer had been conducted. Scale bars are 20µm. Aerobic samples are seen on the left, while the images on the right are from anaerobic microcosms - note the difference in background colour, potentially from the difference in the media for the two conditions, as was observed throughout the experiment.*

6.3.11 Community profiling through DGGE

DGGE (denaturing gradient gel electrophoresis) was employed in order to get a snapshot of the community diversity before the first transfer, to ensure that a reasonably diverse community exists, and that it had not developed into a culture with only a few strains. It is a microbial fingerprinting method which can be used to quickly attain a profile of microbial community diversity. DGGE separates amplicons of approximately the same size based on sequence properties. Denaturation is a chemical process by which proteins and nucleic acids lose their quaternary, tertiary and secondary structure which exists in their natural state.

Aliquots were removed for DGGE analysis on June 9, 2015, about five weeks since

the experiment had been set up, in order to get a snapshot of the community diversity at that time. From each microcosm, 250μl of liquid and 250mg of rock was removed and placed in a sterile Eppendorf tube. 500mg of the initial soil inoculum was also prepared for DNA extraction together with the samples. The tubes were stored at 4°C overnight before DNA extraction the next day.

The day after removing aliquots from each sample DNA extractions were performed, using the FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. For the lysing step, a FastPrep FP120 (Qbiogene, Carlsbad, CA, USA) was used, at setting 6.0 for 40s. For the binding step, the tubes were inverted by hand for three minutes. The DNA was eluted into 100μl of DES(DNase/Pyrogen-Free Water), after being incubated at 55°C for 5mins in a heating block before elution. DNA concentrations were measured with a NanoDrop Lite (Thermo Scientific, Waltham, MA, USA). The samples were stored overnight in the freezer at −20°C.

PCR (polymerase chain reaction) was conducted using forward primer F357GC and reverse primer R518 (see Table 6.2), GoTaq G2 Colourless Master Mix and MQ water. A 25μl reaction per sample was prepared using the GoTaq G2 Colourless Master Mix according to the proportions presented in Table 6.3. PCR runs were performed on a thermocycler (G-Storm GS1, Gene Technologies Ltd., Braintree, UK) according to the protocol outlined in Table 6.4. The PCR products were run on a 1.2% agarose gel stained with SYBR Gold (Life Technologies, UK) to confirm the presence of extracted DNA in all samples before DGGE was performed. Samples were stored in the freezer at −20°C.

Table 6.2 *Primers used for checking DNA extraction yield before DGGE*

Type	Gene	Forward name	Forward sequence	Reverse name	Reverse sequence
Bacterial	16S	F357GC	5-GC-clamp-GCCTACGGAGGCAGCAG-3	R518	5-ATTACCGCGGCTGCTGG-3

Table 6.3 *Reagents used for in-house PCR*

Reagent	Amount
Forward primer (0.01mM stock)	1.0μl
Reverse primer (0.01mM stock)	1.0μl
Master Mix	12.5μl
MQ	9.5μl
Sample	1.0μl
Total volume	25μl

DGGE was performed with the help of Susana Direito from the School of Physics and Astronomy at The University of Edinburgh, according to a protocol by the

Table 6.4 *PCR program for in-house PCR*

Step	Temperature	Duration	Number of repeats
Initial denaturation	94°C	4 min	
Denaturation Annealing Elongation	94°C 54°C 72°C	0.5 min 0.5 min 0.5 min	35 times
Final elongation	72°C	5 min	
Cooling	4°C	10 min then hold	

Molecular Cell Physiology group at Vrije Universiteit Amsterdam [181].

6.3.11.1 DGGE results

The results from the DGGE can be seen in Figure 6.10. Only the samples that had amplified successfully during PCR were run on the DGGE gel. The samples were, as can be seen in the gel image from left to right: 1 sample of granite anaerobic, 2 samples of gabbro anaerobic, 3 samples of granite aerobic and 1 sample of gabbro aerobic. It can be observed that the communities appear more similar according to the presence or absence of oxygen, than within the rock types. In other words, the communities in the granite aerobic and gabbro aerobic microcosms appear to have some similarities, while there are also similarities between the granite and gabbro anaerobic microcosms. These results were not analysed further, but the snapshot was used as an indicator that the communities were diverse enough to proceed with the first transfer.

6.3.12 Experimental Timeline

After the final transfer was conducted on the 22 September 2015, the actual experiment had effectively started, and four sampling points were conducted at 1, 3, 5 and 7 weeks.

6.3.13 Sampling for each time point

After the samples had been inoculated for 1, 3, 5 and 7 weeks, aliquots were removed from each microcosm for analysis. For each time point, aliquots were removed for pH and ICP-OES analysis, and for the final time point additional

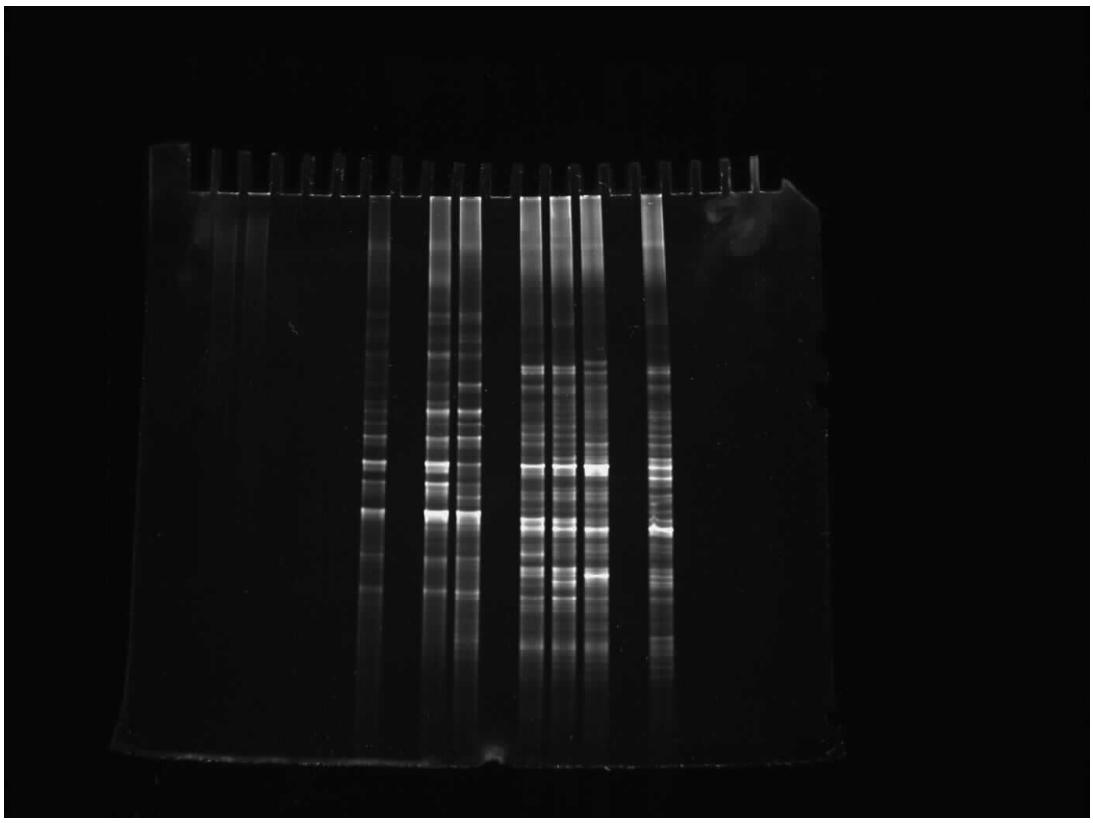


Figure 6.10 *DGGE gel showing community diversity in the microcosms just before the first transfer. From left to right: 1 sample of granite anaerobic (lane 6 from left), 2 samples of gabbro anaerobic (lanes 8-9 from left), 3 samples of granite aerobic (lanes 11-13 from left) and 1 sample of gabbro aerobic (lane 15 from left). Negative control is in the far right lane (lane 19).*

aliquots were removed, as outlined in Section 6.3.13.1.

At each of the first three time points, 4.2ml of liquid was removed from each bottle (using glass pipettes for aerobic samples and needles for anaerobic samples). Then, 200μl was placed into a sterile Eppendorf tube for pH measurements and the remaining 4ml used for ICP-OES. pH of samples was measured in the 200μl aliquots in the Eppendorf tubes using a small probe (Omega, UK).

For the ICP-OES analysis, the 4ml of liquid was filtered it into a 15ml Falcon tube, using a 0.22μm filter. The aerobic samples had been taken out with a pipette, so the liquid was first put into a sterile Petri dish and then taken up with the syringe before adding the filter and filtering it into the Falcon tube. After this, 100μl of 38% HNO3 were added, to a final concentration of 1.5% HNO3, to all samples. In total, 24 samples per time point were prepared for ICP-OES, plus 1 tube with 4ml water and nitric acid as an additional negative control. The

samples were delivered to Lorna Eades at the School of Chemsitry, who helped perform the analysis on a Perkin Elmer Optima 5300 DV ICP-OES machine.

6.3.13.1 Sampling at final time point - end of experiment

For the final time point, extra measurements were added in addition to those already listed above in Section 6.3.13. Firstly, 200μl was removed for pH analysis, and 4ml for ICP-OES. For the DNA extraction, 250mg rock was weighed out and placed into a sterile 2ml Eppendorf tube. In addition, 250μl liquid was removed and placed into the same 2ml Eppendorf. The samples were stored frozen at -80°C until DNA analysis was performed. For microscopy on the supernatant, 200μl liquid was removed from each microcosm and placed in a sterile Eppendorf tube. It was desirable to carry out microscopy on the rocks as well, hence 200mg of rock was taken out and placed in a sterile Eppendorf. To analyse these, 200μl of fresh medium was put in and the rocks and shaken and vortexed gently, before analysed through fluorescence microscopy as detailed in Section 6.3.8. From the aerobic tubes only, 110μl of liquid and 110mg of rock were removed and placed in a sterile Eppendorf tube for plating on 0.2% yeast extract plates (2g/L yeast extract (Oxoid, UK), 20g/L agar bacteriological no.1 (Oxoid, UK)). Aliquots of 1ml were removed from the anaerobic microcosms for sulfide analysis, as the presence of sulfides in the anaerobic microcosms was suspected from the smell when using needles to extract liquid, as well as black precipitates forming around the side of the glass in the granite microcosms.

6.3.14 pH

pH is measured to allow determination of acid production. Acidity is a factor which greatly enhances weathering [16]. The generation of acidity in the presence of glucose and NH_4, both of which are constituents of the medium in this study, can be described through [280]:

$$11C_6H_{12}O_6 + 24O_2 + 8NH_4^+ + 8HCO_3^- \longrightarrow 8C_5H_8O_2N + 34CO_2 + 54H_2O \quad (6.1)$$

It is also possible that the acidity originates from the production of organic acid. Specifically, under conditions with low nutrient availability in which glucose is supplied, some organisms can partially oxidise glucose to gluconic acid. When glucose levels have been completely depleted, microorganisms can respire

gluconate to CO_2. Wu *et al.* (2008) [280] found evidence for pH lowering both through generation of gluconic acid and H^+ extrusion from the use of NH_4 as a N source. Changes in pH seen can be both caused by and have an effect on a changing microbial community structure. In natural soils with natural microbial communities, many different organics acids are typically present [278], [92].

pH was measured using a pH probe (Omega, UK) on a sacrificial aliquot of each sample. The pH of each sample was measured in duplicate, and results averaged over the triplicate samples for each condition.

6.3.15 Microscopy Cell Counts

Cell counting enabled quantification of the amount of growth at different time points and establishment of growth curves. Anaerobic growth is generally slower than aerobic growth, and cell counts enable this effect to be observed, if present.

An aliquot of 200μl from each sample was imaged under the microscope for cell counts. Staining was done from a x20 working stock solution of SYBR Gold DNA binding dye (Life Technologies, UK) by adding 10μl of stain to 200μl of sample liquid. Samples were incubated in the dark for 15mins before being imaged. The cells were fixed on a 0.2μm polycarbonate filter paper (Merck Millipore, UK) by washing the 210ul of suspension using a vacuum pump (Fisher Scientific, UK). The filter paper was placed on a glass slide and a droplet of antifade was added to the top of the filter before adding a cover slip on top. The cells were imaged using a Leica DM4000B microscope (Leica Camera AG, Germany) at x100 magnification under blue light from fluorescence prism I3.

6.3.15.1 Microscopy Cell Counts on rocks

For imaging the rock substrate, some additional steps of preparation were performed, by adding 200μl of fresh medium to the rocks, and shaking and vortexing gently, before analysis through fluorescence microscopy as detailed in 6.3.15.

6.3.16 ICP-MS Treatment

Elemental concentrations were measured through ICP-OES (inductively coupled plasma optical emission spectroscopy) in order to attain an insight into the extent of weathering activity in the system. Elements of particular importance are Mg and Ca, which are primary contributors to CO_2 consumption in the carbonate-silicate cycle. Other elements of interest are F, K, P, Na, Si, Sr, Al and Ba. Of these, Fe, Ca, Mg, P, K and Na are major cations from rocks and known to be important for life. Si and Al are cations that are not used by life and are unlikely to be taken up into the cells, and hence are good indicators of the abiotic weathering, as the concentration that is measured in the medium matches that leached from the rock. Thus, for Al and Si, the measured concentrations have not been affected by the biology in the system, which may be the case for other elements that can be taken up into the cells. Zn, Mn, Ni, and Ti are important minor elements that are used by life.

For the ICP-OES analysis, 4ml of liquid was filtered it into a 15ml Falcon tube, using a 0.22μm filter. The aerobic samples had been taken out with a pipette, so the liquid was first put into a sterile Petri dish and then taken up with the syringe before adding the filter and filtering it into the Falcon tube. After this, 100μl of 38% HNO3 were added, to a final concentration of 1.5% HNO3, to all samples. In total, 24 samples were prepared for ICP-OES from the final time point, plus 1 tube with 4ml water and nitric acid as an additional negative control. The samples were delivered to Lorna Eades at the School of Chemsitry, who helped perform the analysis on a Perkin Elmer Optima 5300 DV ICP-OES machine.

6.3.17 CFU abundances and colony types from plating

In order to measure the biomass in each sample, it was desirable to create agar plates for quantifying the number of colony forming units (CFU) in each sample. These types of findings corroborate the biomass estimates from microscopy cell counts, but only the part of the community that is culturable on solid media will be observed. The technique is useful for comparing the relative biomass between samples, as with these types of soil communities there is no *a priori* reason why the culturable portion of the community would differ between the samples.

Agar plates were prepared using the same concentration of nutrients as in

the liquid medium as described in Section 6.3.6, Table 6.1, and 20g/L agar bacteriological no.1 (Oxoid, UK) was added to make 2% agar plates. Crushed, sterile rock powder, which had been milled by hand using a pestle and mortar, was added to the medium, to a final concentration of 0.5g/L. The media were then treated the same was as the liquid media for aerobic and anaerobic conditions, as described in Section 6.3.6, and then autoclaved. Once the media was cooling down after autoclaving, plates were poured and left to stand. The next day, the plates were inspected and it was found that the granite plates had failed to set, both under aerobic and anaerobic conditions. This was presumed to be because the addition of rock powder had lowered the pH to around 3.0, which is below the limit where agar can set. As this problem was difficult to overcome, rock plates were not used further to quantify biomass in this experiment.

6.3.18 DNA Extraction

DNA extraction was performed using the FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. For the lysing step, a FastPrep FP120 (Qbiogene, Carlsbad, CA, USA) was used, at setting 6.0 for 40s. For the binding step, the tubes were inverted by hand for three minutes. The DNA was eluted into 100μl of DES(DNase/Pyrogen-Free Water), after being incubated at 55°C for 5mins in a heating block before elution. DNA concentrations were measured with a NanoDrop Lite (Thermo Scientific, Waltham, MA, USA). The samples were stored overnight in the freezer at -20°C.

6.3.19 PCR

PCR (polymerase chain reaction) was conducted the next day, using forward primer 27F and reverse primer 1389R (Table 6.5). A 25μl reaction per sample was prepared using the GoTaq G2 Colourless Master Mix according to the proportions presented in Table 6.6. PCR runs were performed on a thermocycler (G-Storm GS1, Gene Technologies Ltd., Braintree, UK) according to the protocol outlined in Table 6.7. The PCR products were run on a 1.2% agarose gel stained with SYBR Gold to confirm the presence of extracted DNA.

Table 6.5 *Primers used for checking DNA extraction yield*

Type	Gene	Forward name	Forward sequence	Reverse name	Reverse sequence
Bacterial	16S	27F	AGAGTTTGATCMTGGCTCAG	1389R	ACGGGCGGTGTGTACAAG

Table 6.6 *Reagents used for in-house PCR*

Reagent	Amount
Forward primer (0.01mM stock)	1.0μl
Reverse primer (0.01mM stock)	1.0μl
Master Mix	12.5μl
MQ	9.5μl
Sample	1.0μl
Total volume	25μl

6.3.20 16S rRNA Sequencing

6.3.20.1 External PCR for sequencing preparation

In order to prepare the samples for sequencing, PCR was performed at RTL Genomics, Lubbock, TX, USA, according to a two-step process. Samples were prepared with (5-3) the Illumina i5 sequencing primer (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG) and the gene specific (16S or 18S) primer as the forward primer, and for the reverse primer with (5-3) the Illumina i7 sequencing primer (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG) and the gene specific (16S or 18S) primer. For bacterial 16S rRNA amplification, primers 28F (GAGTTTGATCNTGGCTCAG) and 388R (TGCTGCCTCCCGTAGGAGT) were used. For archaea, archaeal primers 517F (GCYTAAAGSRNCCGTAGC) and 909R (TTTCAGYCTTGCGRCCGTAC) were used (Table 6.8).

PCR was performed on a ABI Veriti thermocyclers (Applied Biosytems, Carlsbad, California) according to the reaction concentrations in Table 6.9 and cycling conditions in Table 6.10.

Table 6.7 *PCR program for in-house PCR*

Step	Temperature	Duration	Number of repeats
Initial denaturation	94°C	4 min	
Denaturation	94°C	0.5 min	35 times
Annealing	54°C	0.5 min	
Elongation	72°C	0.5 min	
Final elongation	72°C	5 min	
Cooling	4°C	10 min then hold	

Table 6.8 *Primers used for Illumina MiSeq*

Type	Gene	Forward name	Forward sequence	Reverse name	Reverse sequence
Bacterial	16S	28F	GAGTTTGATCNTGGCTCAG	388R	TGCTGCCTCCCGTAGGAGT
Archaeal	16S	517F	GCYTAAAGSRNCCGTAGC	909R	TTTCAGYCTTGCGRCCGTAC

Table 6.9 *Reagents used for external PCR for sequencing*

Reagent	Amount
Forward primer (5μM stock)	1.0μl
Reverse primer (5μM stock)	1.0μl
Qiagen HotStar Taq Master Mix	22μl
Sample	1.0μl
Total volume	25μl

6.3.20.2 External 16S rRNA Sequencing

Sequencing was performed using Illumina MiSeq at RTL Genomics, Lubbock, TX, USA, for bacterial and archaeal 16S rRNA.

6.3.20.2.1 Data analysis of bacterial 16S sequencing data. The sequences were analysed in Qiime ([38]), following a standard pipeline (Appendix B) starting with the FASTA files. The sequences were converted to FASTQ files and trimmed of barcodes, and sequences with a Phred score of Q20 or above were retained, where the Phred score measures the likelihood that the nucleotide has been correctly identified [31]. Chimeras were removed using USEARCH61 in accordance with the 97% Silva 119 release and OTUs were defined by open reference OTU picking using the same database, while the remaining sequences were clustered de novo using UCLUST. The resulting tree was used for diversity analyses to a rarefaction depth of 28400 sequences, which was slightly below the sample with the lowest number of sequences. The resulting community composition was displayed in stacked bar charts. Beta diversity analyses were

Table 6.10 *PCR program for external PCR for sequencing*

Step	Temperature	Duration	Number of repeats
Initial denaturation	95°C	5 min	
Denaturation	94°C	30s	35 times
Annealing	54°C	40s	
Elongation	72°C	60s	
Final elongation	72°C	10 min	
Cooling	4°C	Hold	

displayed in PCoA plots.

6.3.20.2.2 Data analysis of archaeal 16S sequencing data. The archaeal 16S data was analysed by Research and Testing Laboratories (Lubbock, TX, USA) according to their pipeline [249].

6.3.21 Measurement of sulfides

Sulfide levels were measured in the anaerobic enrichments, as it was evident during the course of the work that high levels of hydrogen sulfides were present, due to black precipitates forming in the granite anaerobic microcosms. In addition, a distinct sulfide smell was detected from the microcosms while sampling. An assay was used in order to quantify the amount of sulfides in each sample.

6.3.21.1 Sulfide assay

The sulfide assay works by creating a dye that changes colour depending on the concentration of H_2S present in the sample. Changes can then be measured through spectrophotometry. The dye is a mixture of diamine (N,N-dimethyl-p-phenylenediamine sulphate), ferric chloride ($FeCl_3$) and HCl. The sulfide in the samples and standards is immobilised using zinc acetate and acetic acid in a zinc acetate solution. Standards are made up using sulfide crystals that are stored anaerobically, which are made of $Na_2S.9H_20$, to mM concentrations, with zinc acetate solution and dH_20. The zeroth standard is just zinc acetate and dH_2O, and the other standards have zinc acetate, dH_20 and sodium sulfide crystals to the concentrations required.

The protocol used is based on work by Cline [50] and Reese et al. [207]. Firstly, a 100ml zinc acetate solution was made up. This consisted of 100ml water, 9.8g of zinc acetate (powder) (from a concentration of 98g/l) and 0.98ml of acetic acid in liquid form (from a concentration of 9.8ml/l). All parts were added together in the fume hood. Diamine reagent (100ml) was made up by firstly adding 50ml of dH_20 to a bottle. In the fume hood, 50ml of concentrated HCl (at 38% concentration) was put into the bottle. Then, 0.4g (from 4g/L concentration) of N,N-dimethyl-p-phenylenediamine sulfate and 0.6g (from 6g/L concentration) $FeCl_3.6H_2O$ was added. The liquid went yellow as required, and was stored in the dark at 4°C.

Anaerobic water was made up by bubbling dH_20 with N_2 for 1h, and used for making up the standards. Seven bottles were made up with 50ml anaerobic water in each. One bottle was left as a blank. Next, sodium sulfide crystals ($Na_2S.9H_2O$, molecular weight 240.18 g/mol) were dissolved into 50ml of water in a Duran bottle, by adding 0.12g to 50ml to make a 10mM solution. From this, aliquots were added to the different standards into each of 50ml water, as outlined in Figure 6.11. The standards were sealed with butyl rubber bungs and crimped with aluminium seals.

	Molar concentration	Aliquot to add to 50ml water (µl)
Standard 1	1 µM	5
Standard 2	5 µM	25
Standard 3	10 µM	50
Standard 4	20 µM	100
Standard 5	30 µM	150
Standard 6	40 µM	200

Figure 6.11 *Sulfide standards set-up, concentrations referring to 10mM=10000M stock solution of sodium sulfide crystals ($Na_2S.9H_2O$, molecular weight 240.18 g/mol).*

Measurements were conducted using a spectrophotometer (Helios, ThermoScientific, UK). Firstly, blanks were measured, by adding 111μl zinc acetate solution, 1000μl of water and 88μl diamine reagent into an cuvette. The mixture was pipetted up and down to mix properly, and left to stand for 30mins for the reaction to progress and colour to develop. The sample was then placed in the spectrophotometer and absorbance measured at 670nm, after which the spectrophotometer was blanked. Both the blanks and the samples were measured in triplicate.

In order to create the standard curve, standards were set up as described above for the blanks, but with the 1000μl of water replaced by 1000μl of the relevant standard. The absorbance of each of these standards was measured in a cuvette at 670nm in the spectrophotometer.

The actual samples were treated slightly differently, as these came with the potential of some rock powder, which would interfere with the spectroscopy readings. Hence, they were mixed together in an Eppendorf tube, with 111μl zinc acetate solution, 1000μl of sample and 88μl diamine reagent. The mixture was pipetted up and down to mix properly, and left to stand for 30mins for the reaction to progress and colour to develop. The samples were then centrifuged at 10,000g for 2mins, after which 1080μl of the supernatant was placed in a cuvette,

and absorbance 670nm measured in the spectrophotometer.

Finally, the measurements on each sample were repeated without the diamine assay added, in order to measure the intrinsic absorbance of each sample. This was done in order to correct for the fact that the anaerobic medium contained rezasurin, which as a dye has the potential to influence the readings from the spectrophotometer. It was concluded from this that the presence of rezasurin in the medium had not altered the absorbance readings.

6.3.21.2 Sulfide detection via Scanning Electron Microscopy (SEM)

The sulfide deposits in the granite samples were also analysed by SEM-EDS (Scanning Electron Microscopy Energy-dispersive X-ray spectroscopy), in order to attempt to identify the types of sulfides present. Samples of the precipitates were taken from two of the anaerobic granite microcosms and were dried down on glass slides within the anaerobic chamber. The measurements were taken at the SEM facility at the School of Geosciences at The University of Edinburgh, with the aid of Charles Cockell and Toby Samuels. The equipment used was a Carl Zeiss SIGMA HD VP Field Emission SEM (Zeiss, Oberkochen, Germany) and Oxford AZtec ED X-ray analysis system (Oxford Instruments, Abingdon, UK).

6.4 Results

The data gathered in this experiment helps elucidate the weathering process in this system, and show that there are clear distinctions both in the community and leaching patterns between aerobic and anaerobic conditions. Differences in leaching and community are also observed between the two rock types, both under aerobic and anaerobic conditions. Unless otherwise specified, error bars in graphs show the standard error, n=3.

6.4.1 Geochemical analysis - XRF and XRD.

The geochemical analysis, utilising XRF and XRD, confirms that the rocks are representative end-members of intrusive igneous rocks (Figures 6.12 (XRF) and 6.13 (XRD)). From the XRF data, it can be seen that granite has a higher

silica content and hence, in general, lower amounts of other elements and traces, notable exceptions being K, Cu and Ba. Granite has lower concentrations of several biologically important elements, such as Fe, Ca, Na and Mg, and also of Al, which is a major cation not used by life. Of the major elements, granite has a lower concentration of K and P, both of which are elements important for biology.

Majors		Lizard gabbro	Shap granite		Traces		Lizard gabbro	Shap granite
SiO2	(%)	51.82	69.67		Zn	(ppm)	21.1	24.9
Al2O3	(%)	16.50	14.61		Cu	(ppm)	7.8	50.6
Fe2O3	(%)	6.16	2.30		Ni	(ppm)	109.9	12.9
MgO	(%)	7.87	1.27		Cr	(ppm)	283.5	29.9
CaO	(%)	11.01	1.37		V	(ppm)	173.9	39.9
Na2O	(%)	3.38	3.45		Ba	(ppm)	22.5	678.7
K2O	(%)	0.416	5.321		Sc	(ppm)	41.5	4.7
TiO2	(%)	0.525	0.489		La	(ppm)	n.d.	28.9
MnO	(%)	0.095	0.060		Ce	(ppm)	5	60.1
P2O5	(%)	0.035	0.190		Nd	(ppm)	3.5	20.9
LOI	(%)	1.99	1.03		U	(ppm)	0	8.1
Total		99.80	99.75		Th	(ppm)	0.4	15.4
					Pb	(ppm)	2.5	39.3
					Nb	(ppm)	0.9	13.3
					Zr	(ppm)	24.7	120.4
					Y	(ppm)	13.1	10.5
					Sr	(ppm)	307.5	422.7
					Rb	(ppm)	10.7	266.3

Figure 6.12 *Elemental composition from XRF analysis, listed for major and trace elements in the two different rock types under study, Lizard Gabbro and Shap Granite.*

Lizard gabbro			Shap granite	
Mineral	Vol. %		Mineral	Vol. %
Calcite	0.13(15)		Quartz	34.4(39)
Dolomite	2.12(46)		Calcite	0.53(14)
Albite	10.8(15)		Dolomite	0.78(22)
Anorthite	3.1(18)		Pyrite	0.132(74)
illite_90144	1.70(49)		Albite	2.98(75)
"Chlorite IIb"	1.95(72)		Anorthite	1.6(11)
Orthoclase	1.39(34)		Gypsum	0.30(17)
"Ankerite Fe0.7"	1.32(64)		illite_90144	2.91(59)
Actinolite	29.2(29)		"Kaolinite (BISH)"	1.23(34)
"Hornblende magnesian iron"	10.1(12)		"Chlorite IIb"	0.89(52)
Antigorite	0.00(56)		"Microcline maximum"	9.9(12)
Chrysotile	1.39(41)		Orthoclase	8.3(10)
Tremolite	2.64(55)		"Muscovite 2M1"	2.15(61)
"Augite Px"	1.98(43)		"Oligoclase An16"	19.4(24)
"Oligoclase An16"	14.8(19)		"Oligoclase An25"	6.0(12)
"Oligoclase An25"	5.4(26)		"Andesine An50 C-1structure"	5.1(13)
"Andesine An50 C-1structure"	4.9(49)		"Andesine An50 C1structure"	0.00(97)
"Andesine An50 C1structure"	0.0(48)		"Labradorite An65"	0.0(14)
"Labradorite An65"	0.1(19)		"Bytownite An85"	3.2(14)
"Bytownite An85"	7.0(26)			

Figure 6.13 *Mineral composition of the two rocks under study, Lizard Gabbro and Shap Granite from XRD analysis.*

6.4.2 pH

pH in the microcosms was monitored (Figure 6.14). The anaerobic microcosms, including controls, became increasingly alkaline (Figure 6.15). These changes were statistically significant at 0.05 confidence level for granite (t-test, p=0.000864), but not for gabbro (t-test, p=0.105). Contrastingly, the aerobic microcosms with a biological inoculum ended up slightly acidic at about pH 5.5-6, presumably due to build-up of acidic waste products (Figure 6.15). These changes were statistically significant at 0.05 confidence level for gabbro (t-test, p=0.00136) and granite (t-test, p=0.00842). Their respective controls display very little change from their initial pH around 7.5-8.5, although the anaerobic microcosms are seen to increase slightly (Figure 6.16). No significant differences are observed between the rock types under either aerobic or anaerobic conditions at the end of the experiment.

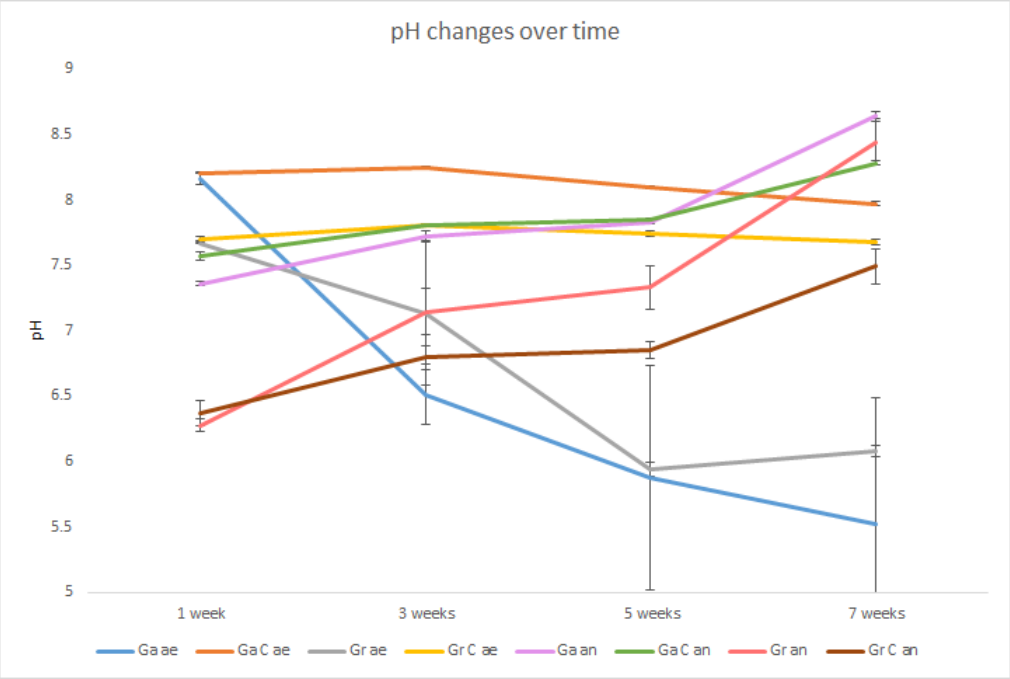


Figure 6.14 *pH changes over time in granite and gabbro aerobic and anaerobic microcosms. Error bars are standard error, n=3.*

6.4.3 Elemental Leaching from ICP-OES

From the ICP-OES data, here presented only as end-points from weeks 1 and 7, some interesting trends can be determined (Figures 6.17, 6.18, 6.19 and 6.20).

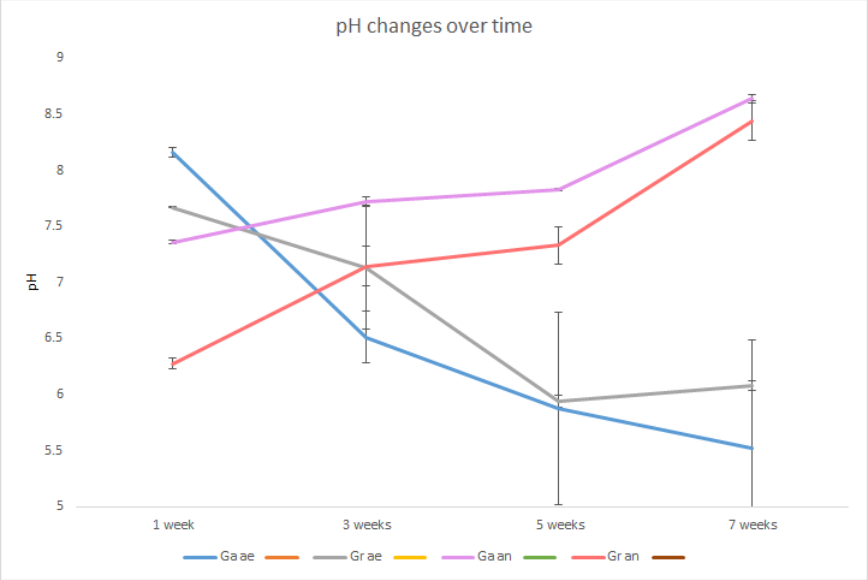


Figure 6.15 *pH changes over time in granite and gabbro aerobic and anaerobic microcosms, showing only the samples without controls. Error bars are standard error, n=3.*

Al is a major cation not used by biology, and as such provides a good way of understanding leaching processes without the influence of uptake by cells. In this study, Al is observed to increase over time under aerobic conditions in both granite and gabbro. For anaerobic granite, a significant decrease of Al leaching over time is observed for both biology (0.05 confidence level, t-test, p=0.00648) and controls (0.05 confidence level, t-test, p=0.0299). There is a significant difference between the rock types under both aerobic (0.05 confidence level, t-test, p=0.000299) and anaerobic (0.05 confidence level, t-test, p=0.000734) conditions at week 1, but not at week 7, so the differences between the rock types decrease over time. There are significant differences between aerobic and anaerobic conditions only for granite at both week 1 (0.05 confidence level, t-test, p=0.000245) and week 7 (0.05 confidence level, t-test, p=0.0206). Significant differences between biology and control are seen for aerobic granite at week 7 (0.05 confidence level, t-test, p=0.0195), anaerobic gabbro at week 1 (0.05 confidence level, t-test, p=0.0479) and week 7 (0.05 confidence level, t-test, p=0.000328), and anaerobic granite at week 1 (0.05 confidence level, t-test, p=0.0260).

Ca is a major cation used by life, and is important for maintaining cell structure and motility, as is an illustrative example of the leaching differences observed. The most striking differences observed for Ca is an increase in leaching over time for both granite (0.05 confidence level, t-test, p=0.00503) and gabbro (0.05 confidence level, t-test, p=0.0314) under aerobic conditions. There are significant

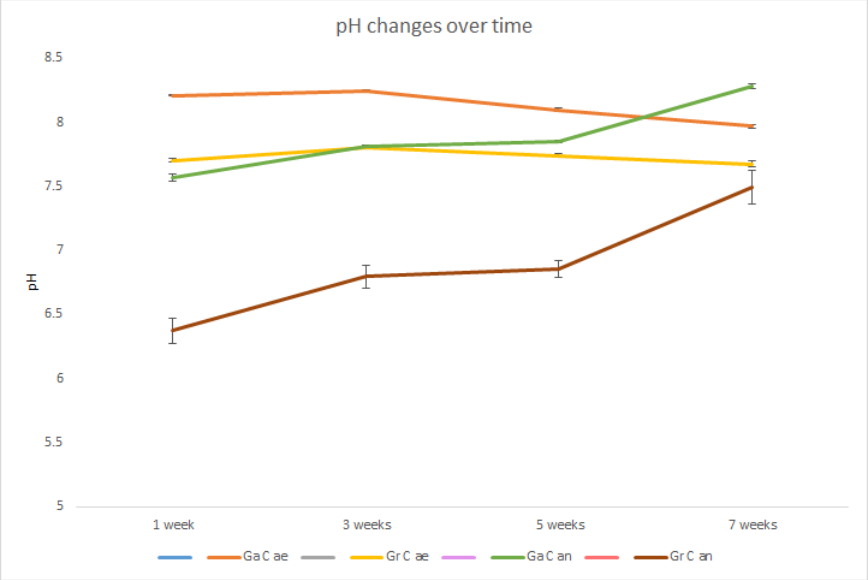


Figure 6.16 *pH changes over time in granite and gabbro aerobic and anaerobic microcosms, showing just the controls. Error bars are standard error, n=3.*

differences between the rock types at both time point: aerobic week 1 (0.05 confidence level, t-test, p=0.000805), anaerobic week 1 (0.05 confidence level, t-test, p=0.0145), aerobic week 7 (0.05 confidence level, t-test, p=0.00519) and anaerobic week 7 (0.05 confidence level, t-test, p=0.000265). There are significant differences between aerobic and anaerobic conditions at both time point: gabbro week 1 (0.05 confidence level, t-test, p=0.000169), granite week 1 (0.05 confidence level, t-test, p=0.00207), gabbro week 7 (0.05 confidence level, t-test, p=0.000797) and granite week 7 (0.05 confidence level, t-test, p=0.00602). The differences between biology and controls are mostly significant, apart from granite aerobic week 1 and granite anaerobic week 7.

For the remaining elements, significance levels can be seen in Figure 6.21. For Fe, large amounts of leaching are seen only for granite anaerobic biology and controls at week 1, which then decrease until week 7. For K, leaching is at similar levels in all microcosms, with slightly more leaching in gabbro than in granite for both aerobic and anaerobic conditions, and leaching increasing in gabbro over time, but decreasing in granite. For Mg and Mn, there is a much higher amount of leaching in granite than in gabbro, for both aerobic and anaerobic conditions. For Na, there is a higher amount of leaching in gabbro than granite, for both aerobic and anaerobic conditions. For Cu and Co, which have similar leaching patterns, there is in general a higher amount of leaching in granite than gabbro. There is an increase in Ni leaching over time at aerobic conditions for both granite

and gabbro. P exists in highest abundance for gabbro controls for both aerobic and anaerobic conditions. For S, in the aerobic microcosms there is a much higher presence of S in granite than gabbro. For the anaerobic microcosms the amount of S decreases over time. The amount of Si increases over time in all microcosms, with the largest increase seen in granite and gabbro aerobic microcosms. Ti levels decrease over time for aerobic microcosms, but increase over time in the anaerobic microcosms.

A summary of the significant differences (confidence level 0.05, paired t-test) can be seen in Figure 6.21. There are differences in leaching for all the elements under study, and leaching patterns differ depending on rock type, whether or not oxygen is present, whether or not biology is present, and also depending on time throughout the experiment. There are clear differences between the leaching pattern of granite and gabbro under both aerobic and anaerobic conditions in most elements under study.

When dividing out the controls (Figures 6.19 and 6.20), the most prominent effects of biological weathering appears to be the increased concentration of Mn, Ni and Si in the gabbro aerobic microcosms, and of Al and Co in the granite aerobic microcosms. For the anaerobic microcosms, the strongest evidence of biological geochemical alteration is a decrease S, and an increase in P for granite and an increase in Ti for gabbro. Comparing the leaching patterns over time, the most obvious patterns are seen for Al (increase in gabbro aerobic, decrease in granite anaerobic), Ca (increase in aerobic samples), Fe (decrease anaerobic granite), K (increase aerobic, decrease anaerobic), Na (increase all samples), Ni (increase aerobic granite and gabbro), S (decrease anaerobic), Si (increase all samples) and Ti (decrease aerobic). Considering whether or not the rock type affects weathering, this can be observed to be the case for Al (anaerobic granite higher than gabbro), Fe (anaerobic granite higher than gabbro), K (gabbro higher than granite), Mg (granite higher than gabbro), Mn (granite higher than gabbro), Na (gabbro higher than granite), Co (granite higher than gabbro) and Cu (granite higher than gabbro). Differences in leaching patterns for aerobic and anaerobic conditions can be observed for Al, Ca, Fe, Na, Ni, S, Si and Ti.

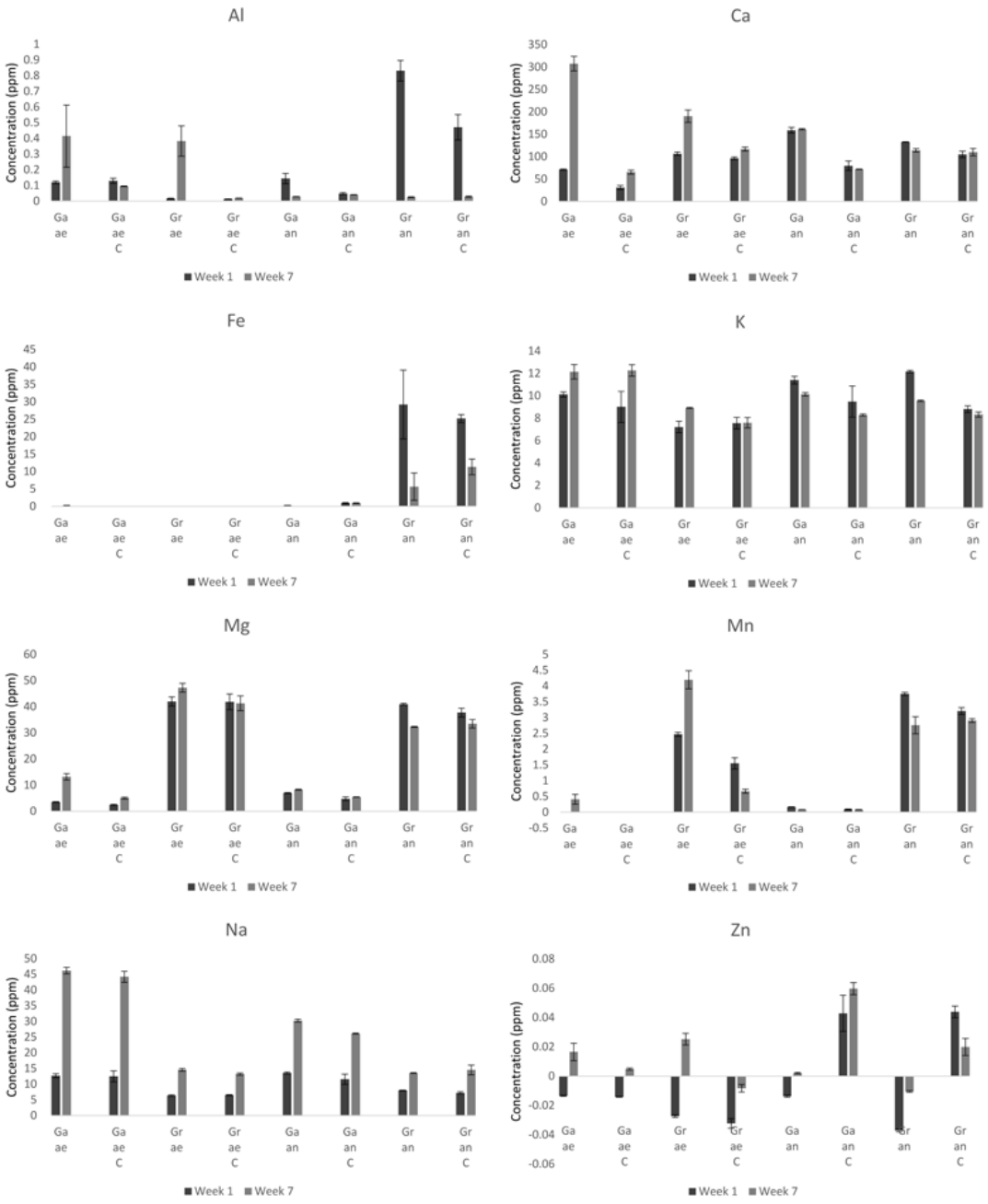


Figure 6.17 *ICP-OES results at weeks 1 (black) and 7 (grey), for Al, Ca, Fe, K, Mg, Mn, Na, Zn. Acronyms used: Ga = gabbro, Gr = granite, ae = aerobic, an = anaerobic, C = control. Error bars are standard error, n=3.*

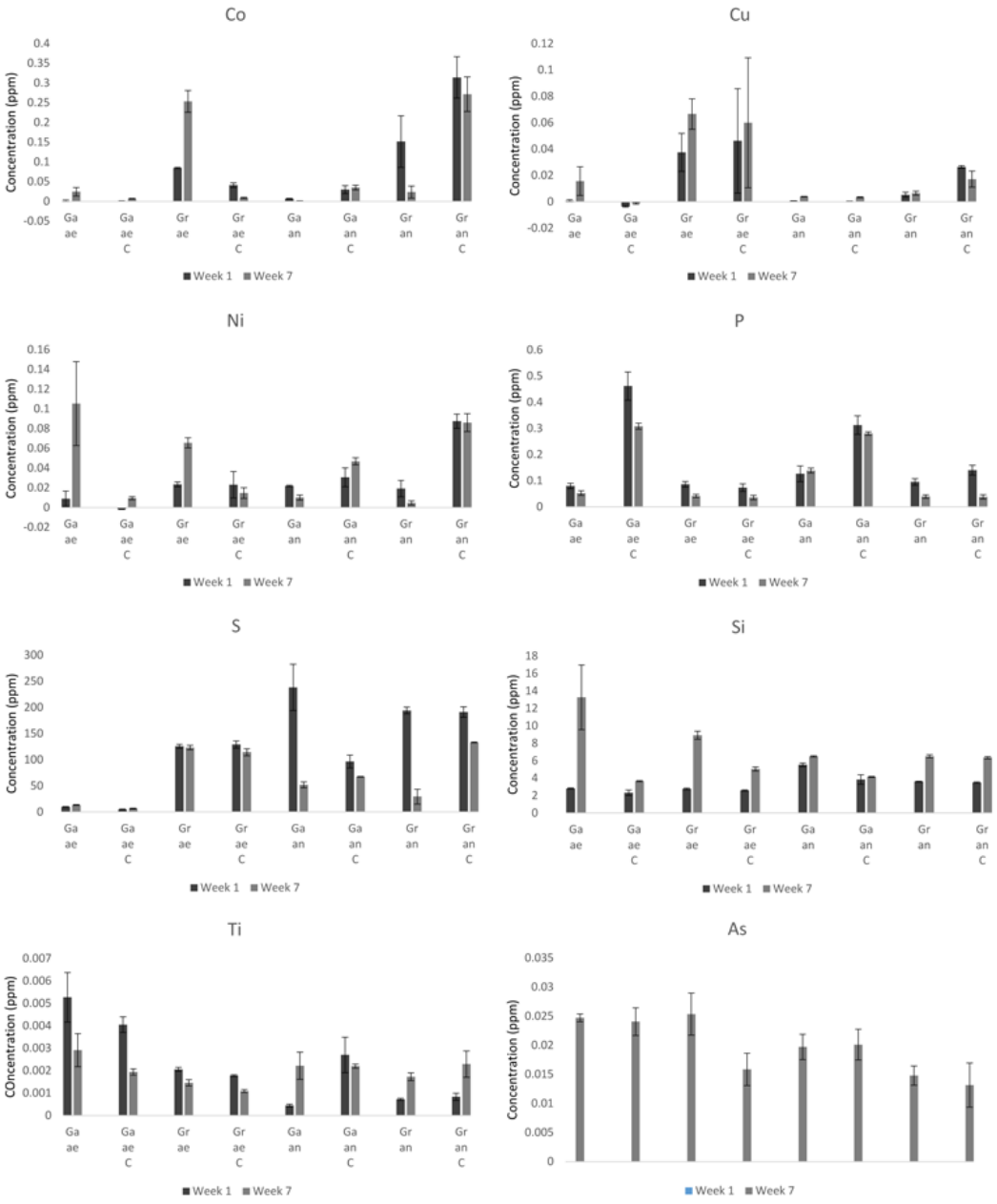


Figure 6.18 *ICP-OES results at weeks 1 (black) and 7 (grey), for Co, Cu, Ni, P, S, Si, Ti, As. Acronyms used: Ga = gabbro, Gr = granite, ae = aerobic, an = anaerobic, C = control. Error bars are standard error, n=3.*

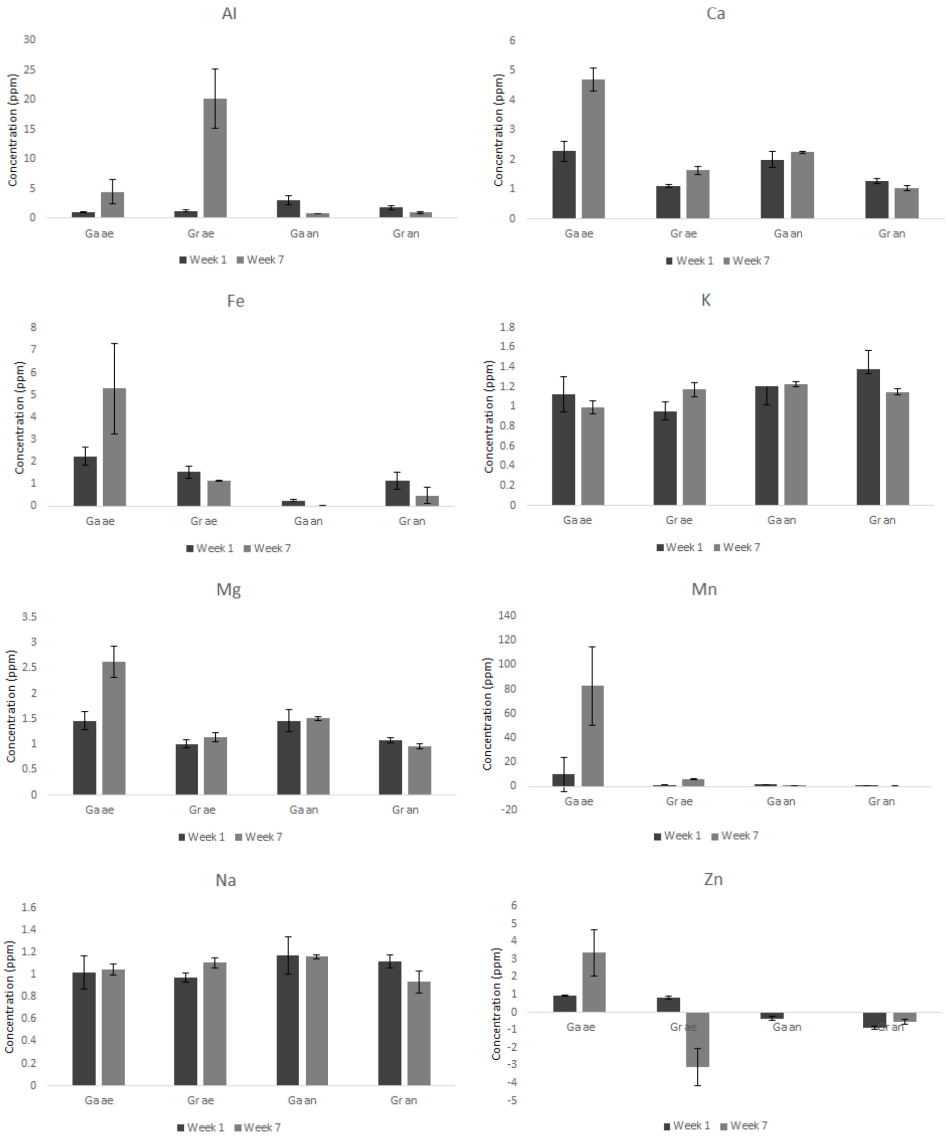


Figure 6.19 *ICP-OES results at weeks 1 (black) and 7 (grey), where sample concentrations have been divided by the respective control concentrations, for Al, Ca, Fe, K, Mg, Mn, Na, Zn. Acronyms used: Ga = gabbro, Gr = granite, ae = aerobic, an = anaerobic, C = control. Error bars are standard error, n=3.*

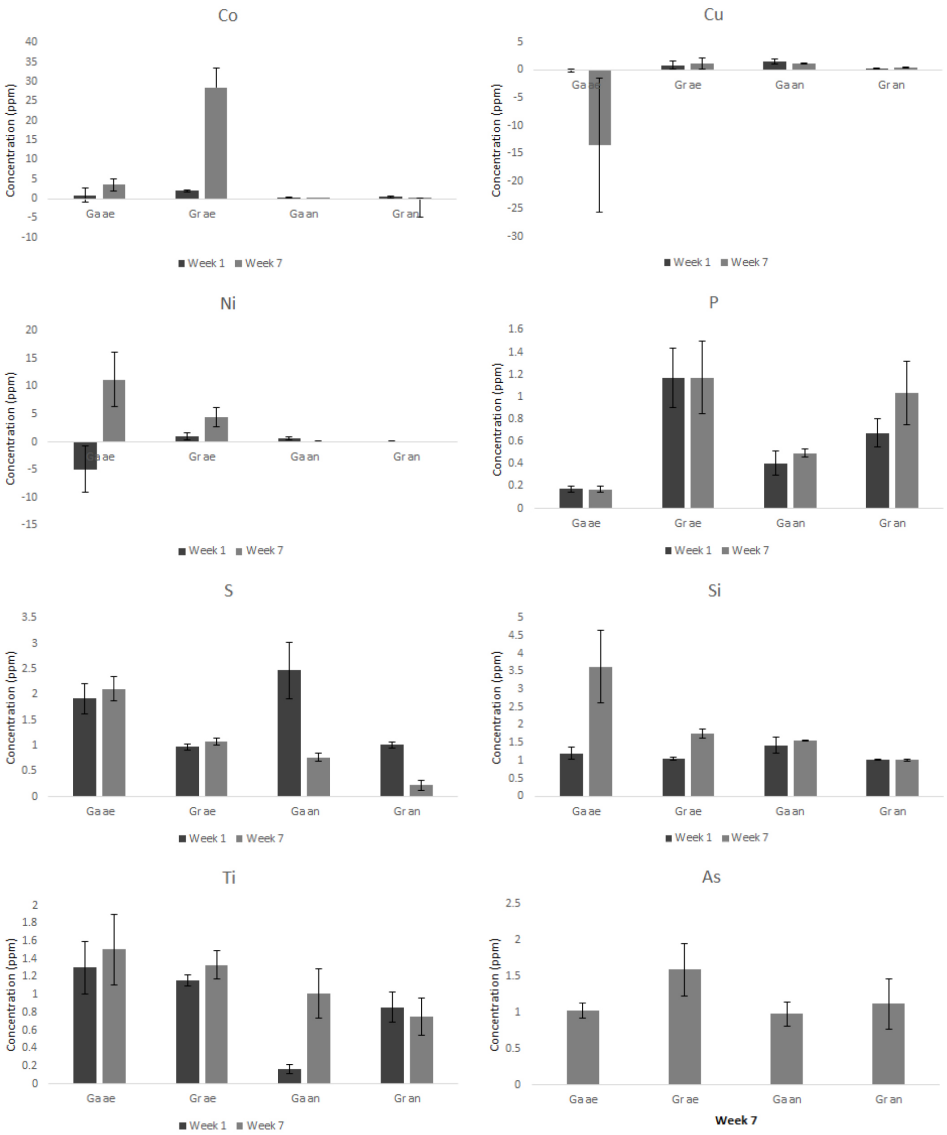


Figure 6.20 *ICP-OES results at weeks 1 (black) and 7 (grey), where sample concentrations have been divided by the respective control concentrations, for Co, Cu, Ni, P, S, Si, Ti, As. Acronyms used: Ga = gabbro, Gr = granite, ae = aerobic, an = anaerobic, C = control. Error bars are standard error, n=3.*

	Al	Ca	Fe	K	Mg	Mn	Na	Zn	Co	Cu	Ni	P	S	Si	Ti	
Time gabbro aerobic		0.00503			0.019336		0.00056	0.039213					0.012032			
Time granite aerobic		0.031448				0.035004	0.004198	0.007819	0.027609	0.024454	0.028802	0.010056		0.007659	0.024589	
Time gabbro anaerobic			0.029948			0.003286	0.00207	0.00063	0.022703	0.005239	0.035801		0.042182			
Time granite anaerobic	0.006475	0.025897		0.000302	0.002182		3.98E-05	0.001009				0.012785	0.002993	0.005788	0.022453	
Rock type aerobic at week1	0.000299	0.000805		0.006684	2.33E-05	2.74E-06	0.000578	0.000102	4.75E-06				5.85E-06		0.044566	
Rock type anaerobic at week1	0.000734	0.014456	0.042366		1.31E-07	2.03E-07	7.07E-05	3.3E-05						0.000876	0.022893	
Rock type aerobic at week 7		0.005187		0.007397	7.88E-05	0.000306	9.3E-06		0.001519	0.032507			1.31E-05			
Rock type anaerobic at week 7		0.000265		0.02549	6.2E-08	0.000608	3.88E-06	6.57E-05				0.00094				
Oxygen gabbro week 1		0.000169	0.005631	0.034326	0.000119	1.18E-05							0.006674	0.000232	0.012095	
Oxygen granite week 1	0.000245	0.002073	0.041473	0.000699		9.02E-05	0.002744	0.001537					0.000675	0.001326	0.000194	
Oxygen gabbro week 7		0.000797		0.038674	0.015708		0.000149					0.002521	0.002419			
Oxygen granite week 7	0.02061	0.006023		0.001495	0.00087	0.021878		0.000868	0.001918	0.00675	0.000401		0.003133	0.007986		
Biology and control for gabbro aerobic at week 1		0.001084	0.010101		0.025369	0.025794				0.014639		0.002281	0.010188			
Biology and control for granite aerobic at week 1						0.00867			0.001873						0.048413	
Biology and control for gabbro anaerobic at week 1	0.047902	0.002841	0.005452		0.037177	0.004219						0.015943	0.036614	0.046342	0.046404	
Biology and control for granite anaerobic at week 1	0.026038	0.017774		0.000441		0.009672				0.000555	0.003503					
Biology and control for gabbro aerobic at week 7		0.000126			0.003031							6.35E-05	0.001407			
Biology and control for granite aerobic at week 7	0.019473	0.007225	0.008318	0.04939		0.000265			0.000915		0.002489			0.001692		
Biology and control for gabbro anaerobic at week 7	0.000328	4.12E-07	0.000237	0.000477	0.000115		0.000998		0.00465		0.001073	0.000226		7.74E-06		
Biology and control for granite anaerobic at week 7				0.007453					0.005828		0.000957		0.001846			

Figure 6.21 *Summary of all significant differences in leaching seen in the weathering experiment, confidence level 0.05, t-test.*

6.4.4 Cell Counts

The cell counts measured at 7 weeks can be seen in Figures 6.22 (logarithmic scale) and 6.23, for both the measurements from the liquid and the rock. From the logarithmic graph an order of magnitude difference between the number of cells in the liquid compared to the rock (10^6 cells/ml vs. 10^5 cells/ml) can be observed. This difference must be treated with some caution, as the effectiveness of the method of removing cells from the rocks is unknown, and it is likely that the sample here only represents a subset of the total community in the rocks.

It is also visible that in the liquid, there is roughly one order of magnitude difference in cell concentration between the aerobic and anaerobic communities (again, 10^6 cells/ml vs. 10^5 cells/ml), consistent with the fact that anaerobic metabolisms often generate less energy and hence the community might grow slower [52], or the environment may sustain a smaller population. These differences between the liquid cell counts for the aerobic and anaerobic communities are statistically significant at the 0.05 confidence level for both gabbro (unpaired t-test, p=0.0352) and granite (unpaired t-test, p=0.0356).

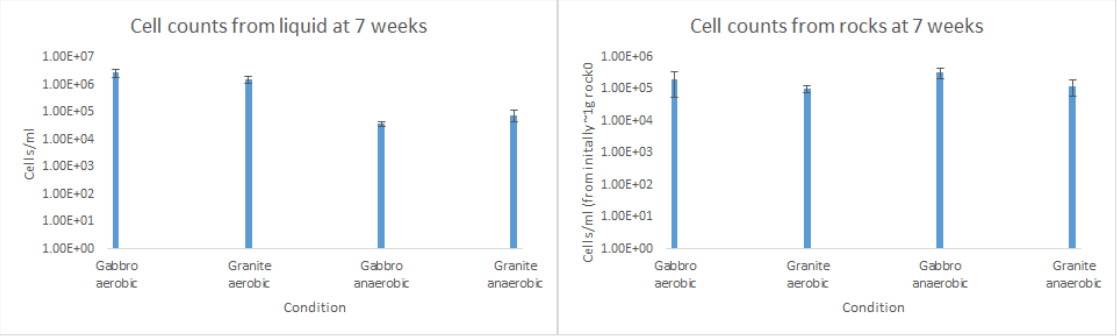


Figure 6.22 *Cell counts for weathering experiment at 7 weeks, measured both in liquid (left) and rock (right) aliquots.*

Looking at the graphs without a log scale (Figure 6.23), it is also observed that the gabbro rocks may contain a higher biomass than the granite rocks, but the uncertainties are large enough that this may be an artifact of the data, and these differences were found to not be statistically significant for the liquid counts or the rock counts.

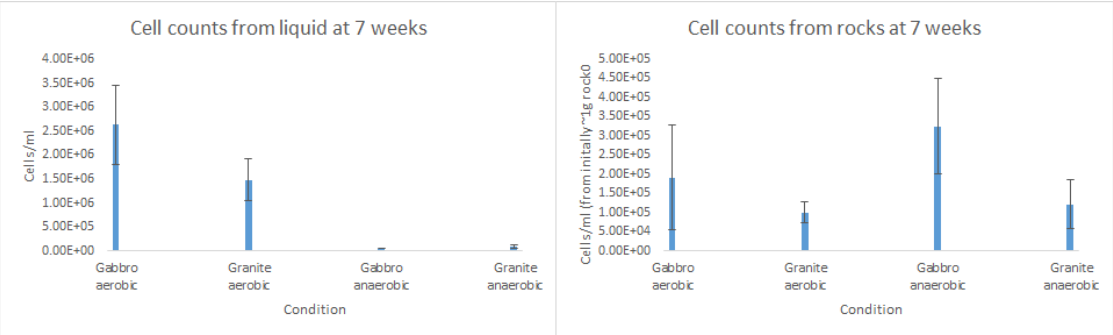


Figure 6.23 *Cell counts for weathering experiment at 7 weeks, measured both in liquid (left) and rock (right) aliquots.*

6.4.5 Community structure

The community structure can be determined from the 16S rRNA sequencing data. An initial soil inoculum was used, so a relatively diverse community of common soil organisms is expected. As the anaerobic granite microcosms failed to amplify in PCR only the data for the three other conditions are shown. The community is dominated by Proteobacteria, with smaller abundances of Bacteroidetes and Firmicutes present in all samples (Figure 6.24). Actinobacteria were observed in some of the aerobic samples, with noticeable abundance in two of the gabbro samples and one of the granite samples. In general, there is a distinction within the communities for each group, even though there are also overarching similarities between each group.

Some more features become visible at class level (Figure 6.25). A large proportion of all samples are Gammaproteobacteria, which dominate the anaerobic gabbro microcosms, making up 58-78% of the community. Betaproteobacteria make up about 50% of the community in the granite aerobic microcosms, while it is 17, 24 and 52% of the gabbro aerobic communities. Alphaproteobacteria are also prevalent in the aerobic microcosms, making up around 25% of the gabbro microcosms and 8, 24 and 41% of the granite microcosms. Actinobacteria make up a small proportion of the aerobic samples.

The anaerobic samples are dominated by Gammaproteobacteria, with smaller proportions consisting of Deltaproteobacteria, Clostridia (Firmicutes) and Bacteroidia (Bacteroidetes) (Figre 6.25).

At genus level, there is less homogeneity within triplicates (Figure 6.26). The anaerobic microcosms are dominated by a large portion of the community

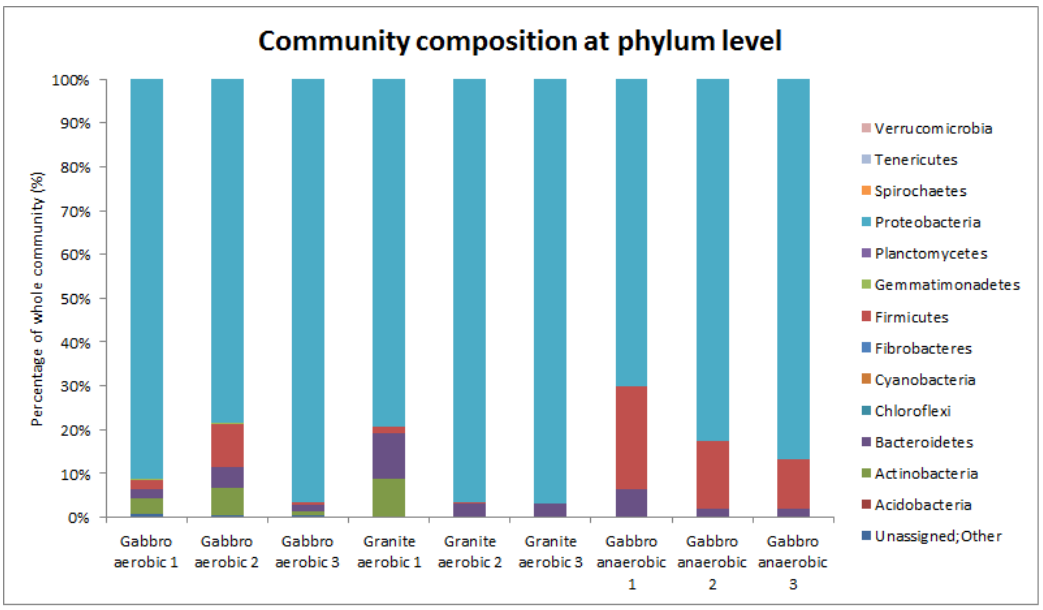


Figure 6.24 *Community structure for weathering experiment at phylum level for all samples, displaying percentages per phylum. Note that the anaerobic granite microcosms failed to amplify for PCR and hence are not included in the analysis.*

belonging to the family Aeromonadaceae. In the analysis carried out by Research and Testing Genomics, this portion is identified as the genus *Tolumonas*. The only taxon identified down to genus level in the in-house analysis here in the anaerobic gabbro is *Desulfovibrio*, while the other strains are only identified at higher taxonomic levels and mostly belong to Clostridia.

The aerobic microcosms at genus level are quite varied, and within triplicates there are clear differences. A significant proportion of both the granite and gabbro microcosms belong to the family Xanthomonadaceae, while the genus is unidentified. Other strains that are observed in both rock types are the family Oxalobacteraceae, the family Nitrosomonadaceae, the family Bradyrhizobiaceae and the order Burkholderiales. In addition, two of the gabbro aerobic microcosms have a significant amount of *Mycoplana*. The only other genus identified which occurs in a large proportion of the relative abundance is *Pelomonas*, which is seen at 7% in one of the gabbro microcosms and 17% in one of the granite microcosms, at below 1% in one microcosm of each rock type, and not observed at all in one microcosm of each rock type.

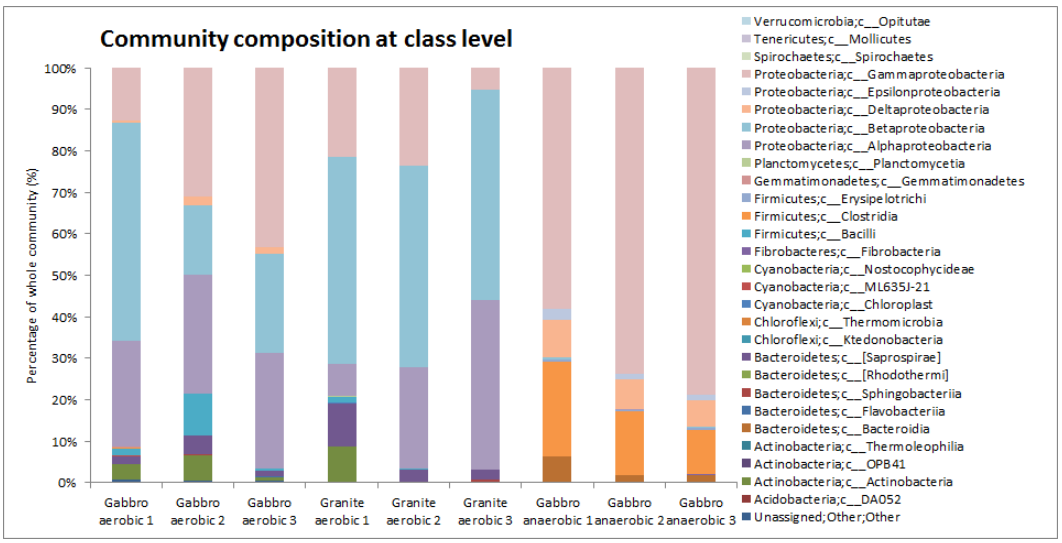


Figure 6.25 *Community structure for weathering experiment at class level for all samples, displaying percentages per class. Note that the anaerobic granite microcosms failed to amplify for PCR and hence are not included in the analysis.*

6.4.5.1 Beta diversity

Beta diversity was analysed using Qiime by producing PCoA plots using Weighted and Unweighted UniFrac, enabling analysis of clustering according to both rock type and time (see Figures 6.27 and 6.28). Each type of community (gabbro aerobic, granite aerobic and gabbro anaerobic) form a separate cluster, indicating that there are distinct differences between the communities for each condition. This applies for both Weighted and Unweighted UniFrac, such that it is true when abundance of different taxa are taken into account, as well as when just presence/absence is recorded.

6.4.5.1.1 Aerobic vs. anaerobic conditions. From this analysis, it is clear that all three communities are distinct from each other. The difference between the aerobic and anaerobic communities can be highlighted by separating the conditions by colour, as seen in Figures 6.29 and 6.30, where aerobic is seen in purple and anaerobic in green.

6.4.5.1.2 Rock type. Similarly, colour-coding the graphs to rock type enables easy visualisation of how the communities differ according to the different rock substrates (Figures 6.31 and 6.32). It is noticeable that there is some overlap between the granite and gabbro aerobic communities using Weighted UniFrac,

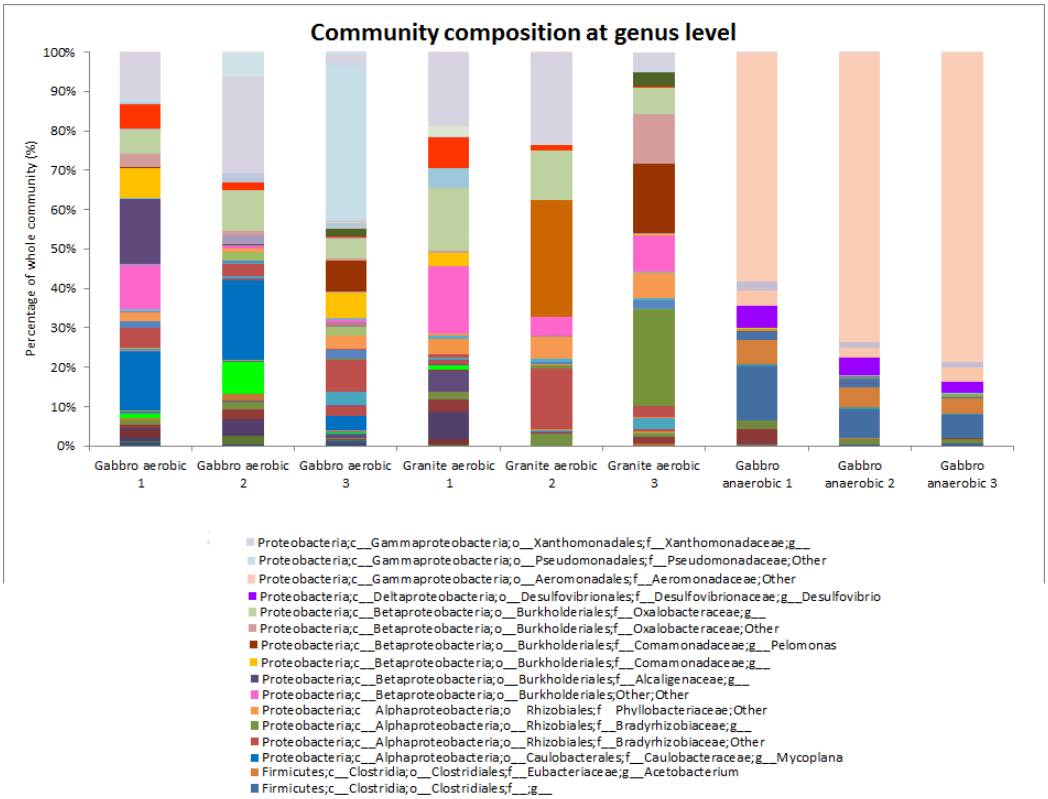


Figure 6.26 *Community structure for weathering experiment at genus level for all samples, displaying percentages per genus. Note that the anaerobic granite microcosms failed to amplify for PCR and hence are not included in the analysis.*

where relative abundance of OTUs is taken into account, and thus that the stronger selection factor for the communities is the presence or absence of oxygen.

6.4.5.1.3 Selection factors. Considering the principal coordinates, it appears that for Weighted Unifrac, when relative abundances are taken into account, there is a stronger separation of the communities by the aerobic vs. anaerobic conditions, than when only considering presence/absence of taxa. This is evidenced by the primary axis at 71.4% and the secondary axis at 9.84%. In comparison, the primary axis for Unweighted UniFrac displays 46.41% of the variation, with the second axis representing 15.76%, indicating that rock type has a stronger effect when only considering the presence or abundance of taxa.

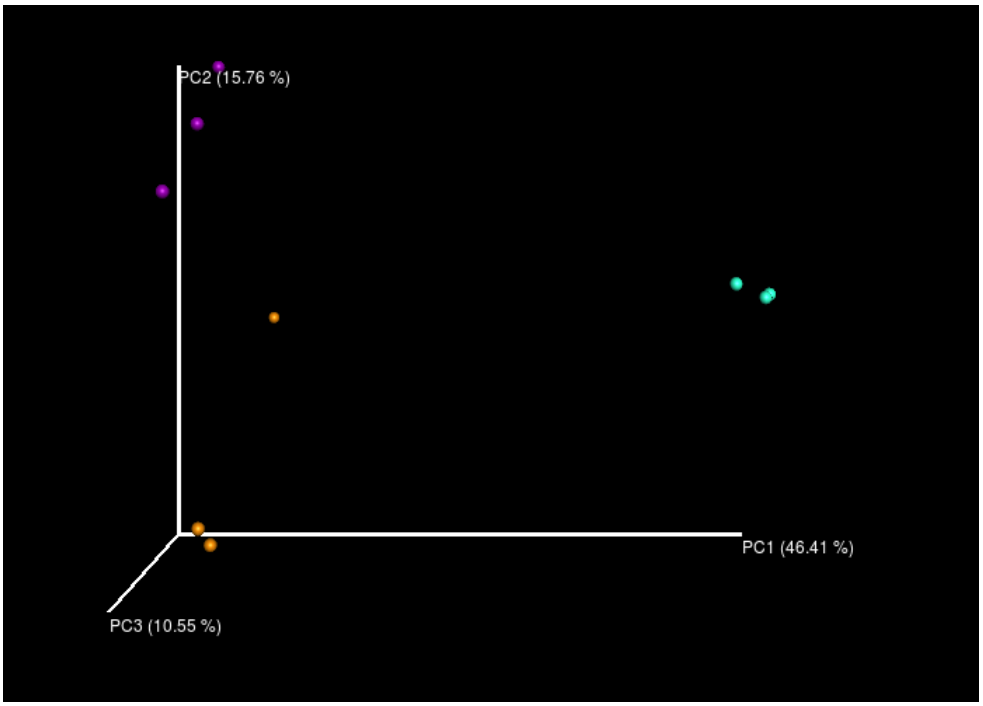


Figure 6.27 *PCoA plot using Unweighted UniFrac. Purple=gabbro aerobic, gold=granite aerobic, cyan=gabbro anaerobic.*

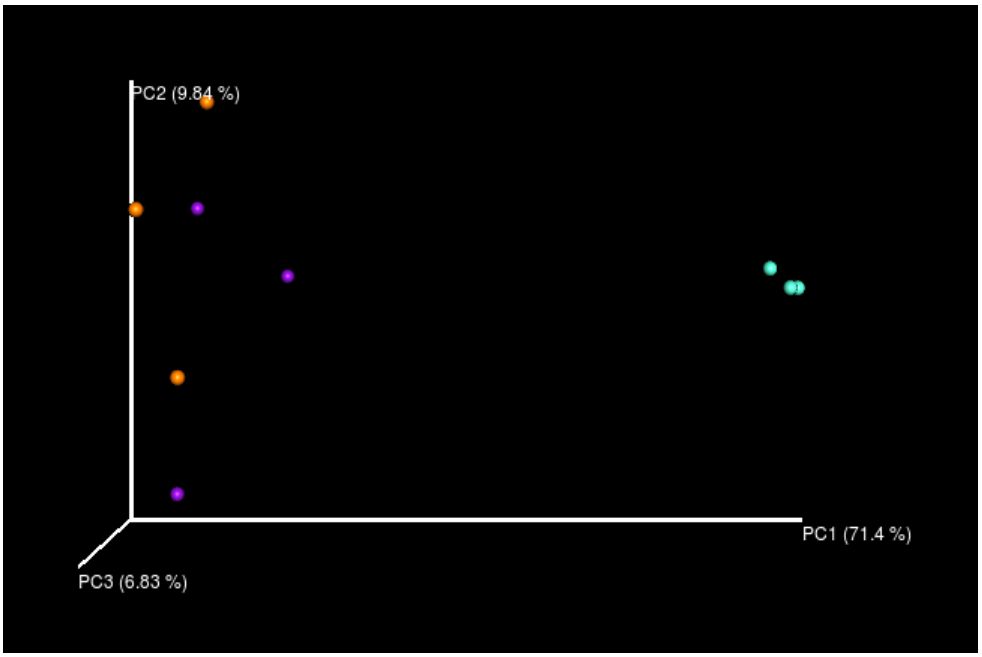


Figure 6.28 *PCoA plot using Weighted UniFrac. Purple=gabbro aerobic, gold=granite aerobic, cyan=gabbro anaerobic.*

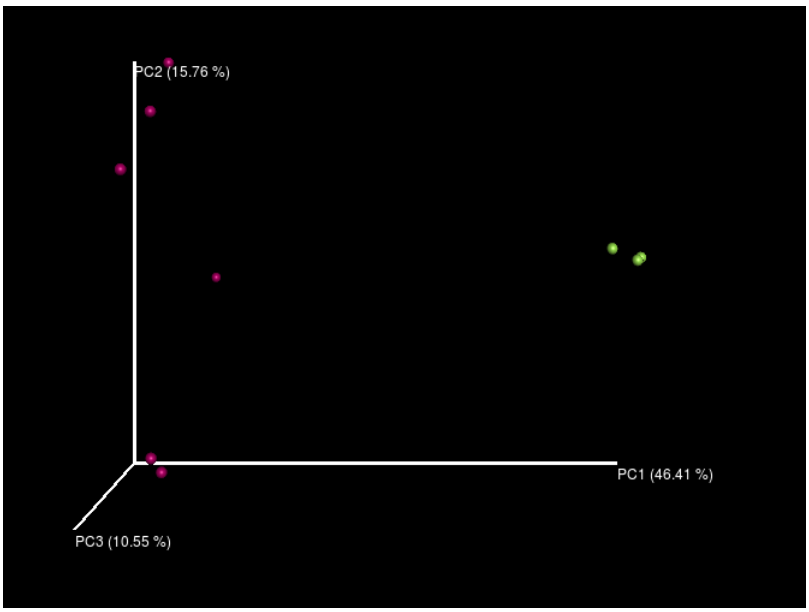


Figure 6.29 *PCoA plot using Unweighted UniFrac. Purple=aerobic, green=anaerobic.*

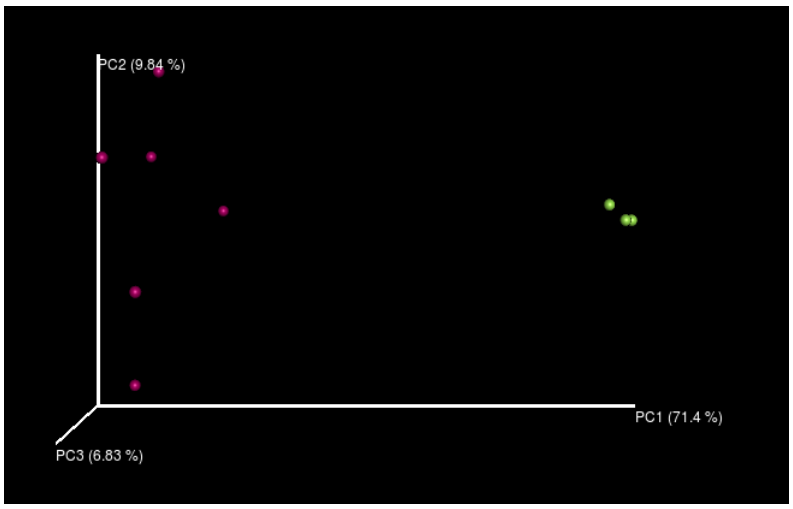


Figure 6.30 *PCoA plot using Weighted UniFrac. Purple=aerobic, green=anaerobic.*

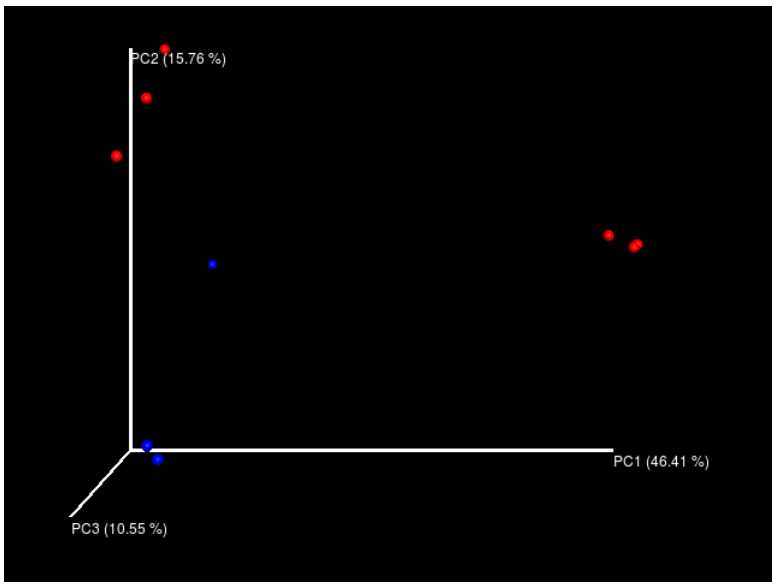


Figure 6.31 *PCoA plot using Unweighted UniFrac. Red=gabbro, blue=granite.*

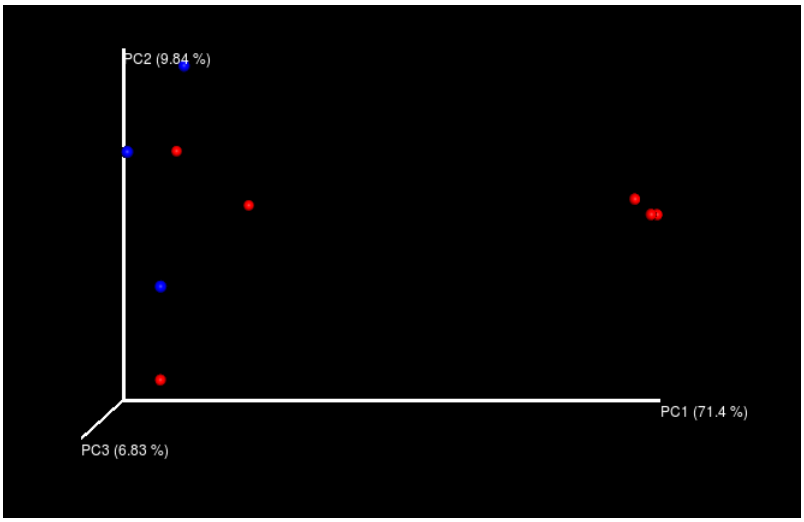


Figure 6.32 *PCoA plot using Weighted UniFrac. Red=gabbro, blue=granite.*

6.4.5.2 Alpha diversity

The number of observed OTUs in this analysis as a function of sequencing depth can be seen in the rarefaction curve in Figure 6.33. This graph gives an indication of whether the communities have been exhaustively sampled - the more of a plateau that is seen, the more likely it is that that sample has been sampled at sufficient depth. The results seen here show that the diversity, as measured by observed OTUs, is highest for the gabbro aerobic samples, followed by the granite aerobic samples, and lastly the gabbro anaerobic samples.

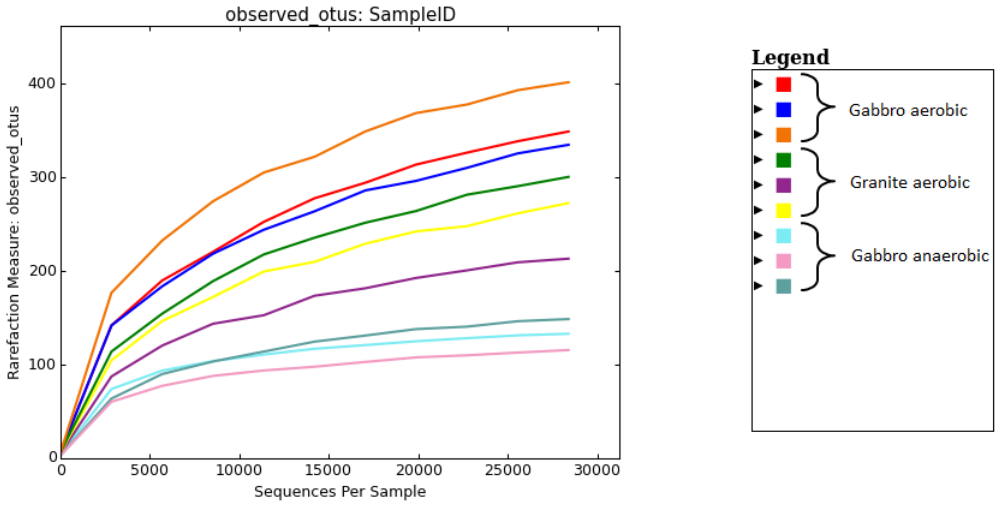


Figure 6.33 *Rarefaction curves showing the number of observed OTUs as a function of sampling depth*

6.4.5.3 Archeal community

Archaeal community identification data was obtained for three samples: one aerobic granite sample and one anaerobic gabbro sample. The two conditions show very different communities, as can be expected from the differing environmental parameters. The aerobic granite community is dominated by *Methanolinea* (orange), followed by *Nitrososphaera* (light green, top) and an unknown genus of Methanomicrobiales (light blue, middle). The anaerobic gabbro community consists largely of *Methanosphaerula*.

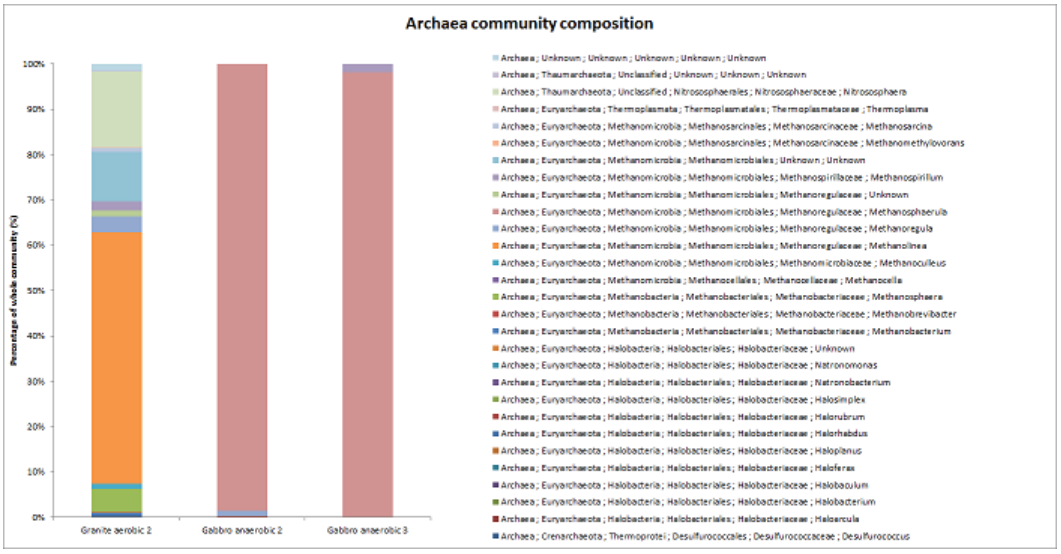


Figure 6.34 *Community composition of archaea from three samples in which archaea were found: one aerobic granite sample and two anaerobic gabbro samples. Dominant taxa: Methanolinea (orange),Nitrososphaera (light green, top), unknown genus of Methanomicrobiales (light blue, middle), Methanosphaerula (anaerobic, pink).*

6.4.6 Sulfide analysis

6.4.6.1 Sulfide assay results

The results of the sulfide assay can be seen in Figure 6.35, showing that the biological microcosms have a much higher presence of sulfides than the abiotic controls. These results are statistically significant at the 0.05 confidence level, for both granite (t-test, p=0.000160) and gabbro (t-test, p=0.0000220). Although black precipitates were only observed in the granite microcosms, the assay shows the concentrations of sulfide to be roughly equal in both the granite and the gabbro microcosms, at about 200μmol/l, with differences not being statistically significant. Thus, although sulfides are present under anaerobic conditions for both rock types, the geochemistries are different such that sulfide only precipitates out in the granite microcosms. The detection of large amounts of sulfide under biological conditions suggests that sulfate-reducing bacteria (SRB) are present in the microcosms [63], [186].

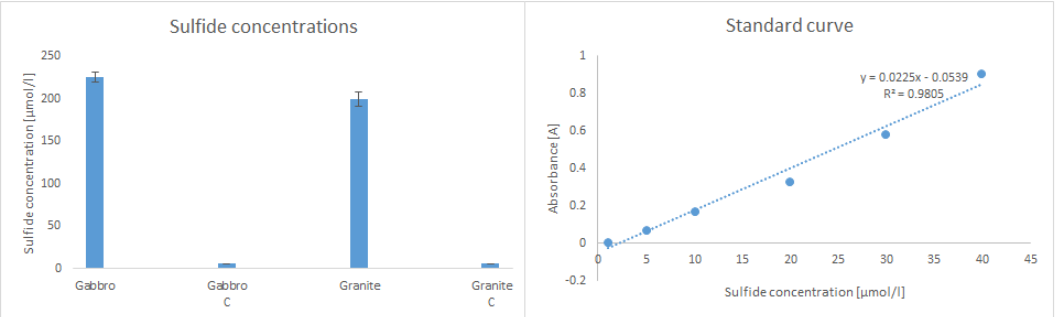


Figure 6.35 *Measurements of sulfides in the anaerobic microcosms at the end-point of the experiment (7 weeks), including the standard curve created.*

6.4.6.2 Sulfides from SEM-EDS results

The results from the SEM-EDS analysis can be seen in Figure 6.36, showing that the most likely candidate for the deposits is FeS_2. This is pyrite, which is the most commonly formed sulfide compound, and hence it is not surprising that it would form in the anaerobic environments in this experiment. Here, S peaks appear with Fe, but peaks are not always at the same stoichiometry. Other elements that are seen in roughly the same stoichiometry include Ca, Al, Cu, Mg and Na, while C, O, Si, Al and Na appear in the spectra without S, as do Ca, K, Fe and Cu to some extent. In three spectra does S appear without Fe, but instead with Ca, K, Na, C and O. The most likely candidate for the majority of deposits is hence FeS_2, but with some possibility that other sulfide compounds may also be present.

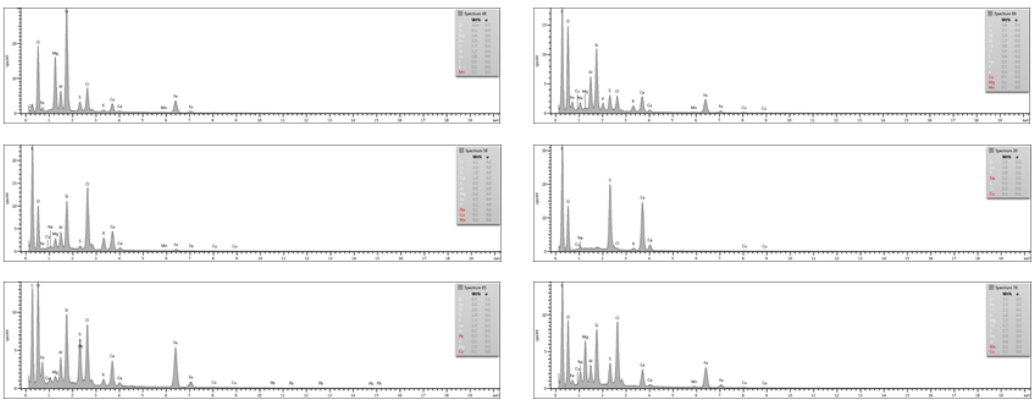


Figure 6.36 *Measurements of sulfide deposits in the anaerobic microcosms at the end-point of the experiment (7 weeks).*

6.5 Discussion

This experiment was set up in order to explore the interplay of biological weathering and microbial community composition, both in the presence and absence of oxygen. Utilising laboratory microcosms with a natural soil inoculum enabled monitoring of geochemical parameters as well as a snapshot of community composition, in order to address the questions posed at the onset. During the course of the experiment, variations in the geochemical environment are observed, as weathering and microbial activity alters the pH and elemental composition of the liquid medium. The microbial community at the end-point of the experiment represents their respective environments while simultaneously bearing testament to their soil beginnings. The communities cluster according to the condition, depending on rock type and whether oxygen is present during growth.

6.5.1 To what extent are some parts of the planetary crust more susceptible to microbial weathering?

In this experiment, microbial weathering was compared and contrasted on two different rock substrates, granite and gabbro. These rocks were chosen as they represent two end-member igneous rock types that make up the majority of the Earth's crust, with granite dominating the terrestrial crust and gabbro the oceanic crust. Thus, the way in which these rocks undergo weathering has a profound impact on numerous important global geochemical cycles. Biological weathering, which is mediated by biological agents, have a role to play in this, as they can accelerate or direct weathering based on their life cycles and characteristics. By monitoring the weathering activity of a microbial community under constrained conditions over a fixed time in the laboratory allows this large-scale phenomenon which takes place over geological timescales to be studied in a useful manner.

Analysing the overall results from the elemental leaching measurements is a complex process, as there are many elements to consider and not necessarily one consistent story for all elements in concert. The main trends that can be observed when it comes to the leaching as depending on rock type are as follows. There is more leaching of Fe, Mg, Mn, Co and Cu observed for granite than for gabbro. Conversely, gabbro seems to have higher concentration of leached Na, P and Ti than granite. For most elements, the weathering profiles are different

for the two rock types. Overall, the granite rock has seen more leaching than gabbro over the course of the experiment. This is the opposite result from that which was hypothesised, namely that more leaching would been seen in gabbro than in granite. Here, it appears as though granite is providing a more diverse geochemical environment with in general higher concentrations of leached elements available.

It is important to consider here what the results actually mean when measuring amount of leached elements through ICP-OES. The results show the concentration of an element as present in the solute, and its presence means that it has not precipitated out or been taken up as biomass. Hence, it is possible that just because the concentration of a particular element is low, it does not necessarily mean that it has not been leached, but can mean that it has merely been used up by biology, bound to biomass, or been precipitated, such that it is no longer detected in the liquid medium [90].

It was hypothesised that there would be different leaching patterns for the two rock types, which was observed. It was also thought that gabbro would exhibit leaching of more elements than granite, but the results support the opposite conclusion, namely that more leaching is observed for granite in this study. The results also support the fact that there is an important biological component to the weathering process, as differences in leaching are seen for the biological microcosms compared to the abiotic controls. Bennett *et al.* (2001) [21] found in their study of weathering using microcosms that biological action can reverse the abiotic weathering sequence, leading to patterns for microbial weathering which would not be expected from a purely geochemical point of view.

A crude comparison of the total amount of leaching can be gleaned by adding up the total concentration of leacheates across all the elements for each condition. If doing this for both rock types including controls, the total amount of leacheate for granite (4160 ppm week 1, 3693 ppm week 7) exceeds that of gabbro (2446 ppm week 1, 29912 ppm week 7) both at weeks 1 and 7. If the controls are not included, granite (2156 ppm week 1, 1835 ppm week 7) exceeds gabbro (1636 ppm week 1, 2026 ppm week 7) at week 1 only, and the differences between the rock types are much smaller. This would suggest that in terms of biological weathering, there is little variation in the overall amounts of leaching for the elements considered.

6.5.2 How does the planetary crust influence the emergent complexity of microbial communities?

In this experiment, the microbial community that emerged after 7 weeks in a weathering microcosm was studied. In terms of community development, this is still a young community, in the early stages of ecological succession, compared to for instance a soil community. As observed in the colonisation study in Chapter 3 this falls in the first regime of community development of the first three to four months, and as such this community may have changed gradually over the next year if left to grow. However, in the colonisation study the community did not change appreciably at phylum level, so it is plausible that this community has already reached the composition it would have had after a year at phylum level.

The communities are similar but distinct between the three conditions for which sequencing data were obtained (Figures 6.27, 6.28, 6.29, 6.30, 6.31 and 6.32). As the samples came from the same or similar inoculum (although the inocula were different for aerobic and anaerobic conditions, they were collected in the same locations), it is not surprising that the final communities have a similar composition under relatively similar laboratory conditions. The conditions do however separate out the communities, so that each condition has its own unique fingerprint on the final community. When considering the relative abundance of each taxa, there is some overlap in the community structure of the aerobic communities for granite and gabbro, however, when looking at only the presence and absence of taxa, the communities all cluster within the triplicates in separate clusters.

Looking at the actual structure of the communities, at phylum level the communities are dominated by Proteobacteria, which are often good colonisers and adapted to living in rocky environments, and are often the first arrivals at for instance fresh lava flows [145]. The second most common phylum is Firmicutes, which are often Gram-positive, spore-forming bacteria found in a range of different environments. The Firmicutes have a higher abundance here in the anaerobic samples. Thirdly, Bacteriodetes form a part of both the aerobic and anaerobic communities, while the Actinobacteria form a small component of the aerobic microcosms only. Bacteroidetes are common in many environments, including soils, and are Gram-negative and non-sporeforming and can be either aerobic or anaerobic. Actinobacteria are important soil organisms that fulfil many functions in soil communities, and often aid the decomposition of organic matter.

Larger differences between the aerobic and anaerobic communities are seen at the class level, where it is noted that the aerobic communities consist mainly of Betaproteobacteria, which are well-known primary colonists that are well adapted to living in nutrient-poor rocky environments [145]. Alphaproteobacteria are the second most common class in the aerobic microcosms, and these are likewise good early colonists. Most nitrogen-fixing bacteria are found amongst the Alphaproteobacteria, and are good primary colonists as nitrogen is often a growth-limiting nutrient in rocky environments, and any species which can fix nitrogen has a distinct advantage to colonise such a habitat [183], [71], [75]. The third largest contingent is made up of the Gammaproteobacteria, which are a diverse class with well-known pathogens and many varied physical adaptations. Gammaproteobacteria are by far the largest portion of the anaerobic samples, which is consistent with the fact that many of these organisms are anaerobic and are often chemoorganotrophs, but can be either photoautotrophs or chemolithotrophs [100]. The anaerobic communities are further made up of Deltaproteobacteria, which contain most of the documented sulfur and sulfate-reducing bacteria and other anaerobes such as iron-reducers, and Clostridia, which are common pathogens, but also found in soils, and are obligate anaerobes.

At the genus level it is noticeable that there is a large amount of variation within triplicate samples from the same condition. Of the three conditions, the anaerobic gabbro microcosms show the most homogeneity, with all three triplicates displaying roughly the same community composition, albeit with some variation in actual percentages. Conversely, the aerobic microcosms of both rock types show a much larger within-triplicate variation, although some genera are seen to have similar percentages across the samples.

Most of the taxa with a high relative abundance in the aerobic microcosms are not classified down to genus level, such as an unidentified genus of the Oxalobacteraceae family, an unidentified genus of the Xanthomonadaceae family, an unidentified genus of the Burkholderia order, an unidentified genus of the Bradyrhizobiaceae family, the genus *Pelomonas* (found in two samples of each rock type), the genus *Mycoplana* (gabbro only) and an unidentified genus of Nitrosomonadaceae. The fact that high relative abundances of around 5-10% are found for only two identified genera is interesting, as it shows that the communities are very diverse with no one genus or species dominating the community, but that the community is made up of many different species all in small abundances. The two genera identified that have larger relative abundances

are *Pelomonas* and *Mycoplana*. *Pelomonas* is a Gram-negative, rod-shaped, non-spore forming bacteria isolated from haemodialysis and industrial water [112], while *Mycoplana* is a Gram-negative, rod-shaped, filament-forming bacteria [269].

For the anaerobic microcosms, the dominant genus is an unidentified member of the family Aeromonadaceae, which are rod-shaped aerobes or facultative anaerobes found in a variety of often aquatic environments, especially freshwater [131], [59]. In the analysis carried out by Research and Testing Genomics, this portion is identified as the genus *Tolumonas*, from which some strains are known to produce toluene in the presence of a carbon source and a toluene precursor, although relatively little is still known about this genus that has been isolated from freshwater sediments [89]. The only genus identified in the in-house Qiime analysis in the anaerobic gabbro is *Desulfovibrio*, while the other strains belong to Clostridia. *Desulfovibrio* are Gram-negative sulfate reducers and are commonly found in soils and aquatic environments, and also in human and animal intestines [110]. *Desulfovibrio* have also been found in deep granitic environments, which is similar to some of the environments here, and thus may have been present in the granitic anaerobic microcosms, although that information was not obtained [199].

Archaea were only possible to amplify and sequence in three samples. Archaea are common in soils, but their prevalence and distribution is not well understood [18], [141], [27]. It is also unknown whether the archaea here are active or inactive in the system, although with the amount of transfer from the original inoculum it seems likely that for archaea to be detected in the final experiment they would have increased in number over time, and hence played an active role in the community. The aerobic granite community is dominated by *Methanolinea*, which are strict anaerobes that produce methane, by using either the organics in yeast extract (when grown in the lab) or acetate for growth. They are often found in sediments, so it is possible that they have grown in anaerobic pockets of the microcosms, although the optimum growth range of 37-50° means that their grown may be unlikely in this set-up [124]. *Nitrososphaera* are ammonia oxidisers, and are often found in soils [242]. The anaerobic gabbro community consists largely of *Methanosphaerula*, which utilise either hydrogen or formate for growth and are often found in fens [230]. The presence of archaea in the system suggests that the community is reasonably diverse, with many potential metabolic pathways available to the community.

It was hypothesised that granite would hold a lower diversity than gabbro, and

the findings from the alphadiversity, looking at observed OTUs, support this. The reasons for the hypothesis, that granite would provide a more nutrient-poor or less diverse environment [190], appear not to be supported, as granite appears to be leaching a broader variety of elements at a higher concentration compared to gabbro.

6.5.3 How would major planetary change, such as the oxidation of the atmosphere, affect microbial weathering?

This experiment enabled conclusions to be drawn regarding how the presence or absence of oxygen would affect microbial weathering processes. Comparing the leaching of elements in the biological microcosms between aerobic and anaerobic conditions for both rock types, we find noticable differences in Al, Ca, Na, Ni, S, Si and Ti for gabbro, and in Al, Ca, Fe, Mn, Cu, Co, Ni, S, Si and Ti for granite. Thus, for the majority of elements, the presence or absence of oxygen changes the effect of microbial weathering, whether that means increasing or decreasing the amount of leaching. For gabbro, anaerobic conditions typically means lower amounts of leaching, apart from for S, whereas for granite it is less clear, although in general by 7 weeks there is less leaching in the granite anaerobic microcosms than the granite aerobic equivalents. These results are consistent with the hypothesis that anaerobic conditions would lead to slower leaching rates, as anaerobic metabolisms are typically slower.

In terms of the community, it was hypothesised that the composition would be different under aerobic and anaerobic conditions. This was indeed found to be the case, although at phylum level these differences are not as strong as they are for the class or genus level analysis, where there are clear differences in most taxa between the communities. The starting communities were also assumed to be different between the aerobic and anaerobic soils, as the soils were sampled separately under aerobic and anaerobic conditions, albeit in immediate proximity of each other. Note that this comparison between aerobic and anaerobic conditions was only possible for the gabbro weathering communities, so it is possible that the situation might have been different for granite. Attempts to extract and sequence the DNA from the initial soil community failed, due to large amounts of humic substances present in the soil that could not be removed during the extraction process.

In accordance with previous literature, anaerobic weathering is found to have different leaching patterns to aerobic weathering, consistent with a different community structure, where metabolisms are generally slower, with higher amounts of acid production, under anaerobic conditions.

6.5.4 How do pioneer microbial communities change or enhance habitability for later colonists?

It is well known that pioneer organisms shape their environment, often making conditions more clement for later arrivals. It was hypothesised that the geochemical composition would be considerably different after seven weeks as opposed to one week, and that the pH in the microcosms would also have altered. The pH was altered for all biological conditions over the 7 weeks of the experiment, with the pH in the aerobic microcosms decreasing by about 2 units from roughly 8.0 to 6.0, and the pH in the anaerobic microcosms increasing from 7.5 to 8.5 for gabbro and from about 6.0 to 8.0 for granite. Thus, as the pH changes in the system, the potential survival of new species, if there had been an inflow of inoculum, would be different at week 7 compared to week 1.

In terms of the leaching data, it was hypothesised that the leached elements would differ at week 7 as opposed to week 1. Differences between weeks 1 and 7 are seen for some elements but not others, and sometimes only for one of the four conditions under study. For aerobic gabbro, significant changes between weeks 1 and 7 in leachate concentration are observed for Ca, Mg, Na, Zn, and S, all observing an increase in leaching. For the anaerobic gabbro microcosms, changes in leaching are seen for Fe, Mn, Na (increase), Co, Cu, Ni (decrease) and S (decrease). For the aerobic granite microcosms, leaching changes are observed for Ca, Mn, Na, Co, Cu, Ni, P, Si and Ti, all increasing apart from P and Ti. Lastly, for the anaerobic granite microcosms, leaching changes are seen for Al (decrease), Ca (decrease), K (decrease), Mg (decrease), Na (increase), P (decrease), S (decrease), Si (increase) and Ti (increase). From these results, it can be noted that there are very different leaching patterns under aerobic and anaerobic conditions, but that the patterns within the aerobic and anaerobic conditions are similar between the two rock types. In other words, the leaching pattern of granite anaerobic and gabbro anaerobic are similar to each other, as are granite and gabbro aerobic to each other. The leaching patterns appear to be dictated by whether the environment is aerobic or anaerobic, whereas rock type

has less influence on the time course of the leaching between week 1 and 7.

This difference is likely caused by the difference in the microbial community between the aerobic and anaerobic microcosms, as the metabolisms present would be different depending on whether or not oxygen is present. This means that the elements that are being used as resources for growth are different, as well as the metabolic byproducts and their effect on the rock substrate. Combined, these effects could create different weathering patterns that would differ according to whether an aerobic or anaerobic microbial community is present.

The results from this experiment thus shows that the microbial community indeed alters the environment away from its original state in terms of pH and geochemical composition through biological weathering of the rock substrate. This means that for a developing community, the pioneer community alters or ameliorates the environment for later arrivals in the process of colonisation, leading to patterns of succession in real communities.

6.5.5 Sulfide production under anaerobic conditions

An analysis was conducted on the sulfide production in the anaerobic microcosms, as black deposits were seen in the granite microcosms. The sulfide assay revealed that sulfides were present in both the gabbro and granite microcosms, but precipitates were only formed for granite. This can be explained by the difference in elemental leaching in the two rock types, and that the precipitates only formed in the granite as there was an available cation for the reaction to take place. The investigations through SEM-EDS show that this is likely FeS_2, which agrees with the ICP-OES data that there is much more Fe available in the granite microcosms than for gabbro. The ICP-OES results for granite show that there is less Fe in the biological microcosms than the abiotic controls, an effect which could be explained if Fe has precipitated out to form FeS_2 complexes. In the gabbro samples, no other cations that readily form sulfide deposits were available in large quantities. Smaller amounts of some sulfide-forming cations were available, such as Zn and Ni, but these were only present in very small quantities, on the same order of magnitude as Fe, at around 0.04 ppm, whereas for granite the concentration of dissolved Fe in the anaerobic abiotic controls was around 30 ppm at 7 weeks.

The detection of large amounts of sulfides in the biological conditions when compared to control conditions indicates the presence of sulfate-reducing bacteria

in the microcosms. These organisms use sulfate as the terminal electron acceptor in the process of degradation of organic carbon, which leads to a production of sulfide as the sulfate is reduced. Sulfate-reducing bacteria are common anaerobic microorganisms, for instance estimated to be responsible for up to 50% of organic carbon mineralisation in marine sediments, hence, their prevalence in these microcosms, which provides an ideal environment for sulfate-reducing bacteria, is not surprising [186].

6.5.6 Comparison with colonisation experiment community

The rocks used in this experiment were the same as those used in the colonisation study, covered in Chapter 3. Hence, it is interesting to compare the microbial communities in these two studies, even though the inocula are vastly different, here being a one-time inoculation from a soil community, whereas in the colonisation study there was a constant influx of microorganisms from the atmosphere into an initially barren environment. There is perhaps little value in comparing with the anaerobic community, but the aerobic environments in the early months may be sufficiently similar to expect some common characteristics within the microbial communities. At phylum level, the communities are similar in the portion of Proteobacteria shared, which dominates the community, and a shared smaller fraction of Bacteroidetes, whereas the other smaller fractions in the colonisation study are Cyanobacteria, and in the weathering study they are Firmicutes and Actinobacteria. At class level, roughly the same splitting between Betaproteobacteria and Alphaproteobacteria is seen for the first few months of the colonisation study as is observed in the weathering experiment. Going down to the genus level, without doing any quantitative analyses, it appears that there are larger differences in the samples between the two experiments, such as for instance that the weathering communities are less dominated by any specific taxa, whereas the colonisation study sees some taxa at a higher abundance in all samples. The conclusion is thus that the rock environments allow for similar microbial communities at the higher taxonomic levels, despite the fact that the inoculation process is very different, and that most of the environmental variables other than the rock substrate are actually different. These last two factors explain the differences seen at the more detailed taxonomic levels.

6.5.7 Difference between triplicate microcosms

As was discussed in Chapter 3, the community in each microcosm is found to be slightly different, even under identical conditions, as can be seen from the PCoA analysis in Figures 6.27 and 6.28, as each triplicate forms a cluster, but the communities do not fully overlap within the triplicate. In this weathering study, small initial differences may have existed in the heterogeneity of the soil inoculum, leading the communities on different trajectories throughout the experiment. Microbial communities are highly complex systems with many components and interactions, and hence small variations at the outset can lead to very different trajectories. This effect has been observed before, where identical microcosms set up as Winogradsky columns with sediment and a natural inoculum led to different community trajectories, with alternative stable states where different microbial phyla dominate the end-point communities after several months [192]. Interestingly, in the study by Pagaling *et al.* (2017) [192], it was found that the communities diverged at a high taxonomic level, with a split seen between communities dominated by either of the phyla Firmicutes or Bacteroidetes, whereas in the weathering experiments conducted in this body of work the microbial communities were dominated by the same phyla, but diverged at genus level. Here, the communities are observed to diverge somewhat under identical conditions, creating distinct communities, while at the same time being constrained by environmental variables leading to broad similarities in community composition.

6.6 Limitations

6.6.1 Lack of sequencing data from all conditions - soil inoculum and granite anaerobic

One drawback of this study which makes it somewhat incomplete is the failure to amplify and sequence the microbial communities in all samples. The two sets of samples that failed were the initial soil inocula and the triplicate anaerobic granite samples. In both instances, it is suspected that interference from humic substances caused the PCR reactions to fail. Without these sets of data, it is not possible to compare the communities with their beginnings, to see how

different they might be from the initial soil inocula. It is also not possible to make comparisons between the communities in the two rock types in the anaerobic case. Despite this, there are several important comparisons that have been drawn using the existing data, and this has enabled addressing all the questions posed at the outset of the experiment. Hence, the missing data was not crucial to achieving the goal of the study, but may have added extra information and insight.

6.6.2 Length of experiment

A potential drawback of the study is the relatively short length of the experiment. This time frame was chosen as it was short enough to get some interesting data, and matched the length of the transfers, which had been shown to generate sufficient diversity in the community from DGGE fingerprinting analysis. Despite this, it is possible that interesting results may have occurred after the experiment was concluded, such as increases in leaching, as weathering and soil formation are typically slower processes taking place over much longer time scales. Although the length of this experiment was sufficient for the aims here, it is possible that new insights may have been generated had it continued, and this may be an area of future exploration that would lead to interesting insights into weathering and, in the long term, soil formation. Results from Chapter 3 suggests that communities may change on timescales of months to years, so it is possible that the microbial community in the weathering microcosms may have continued to change over time if the experiment had been extended.

6.6.3 Functionality analysis and active organisms

One type of analysis not conducted here was functionality assessment, which may have aided addressing deeper questions about the functions available in the system and which organisms were active. The DNA sequencing data only gives an insight into the DNA present, and it is possible that some of the organisms detected are either not active, or do not display their whole range of functions. Key functions to look for here might have been various types of metabolisms known to correlate with the weathering of rock substrates. This analysis could be conducted either through metagenomics or by probing specific functional genes, or using for instance proteomics or metabolomics. This may have led to some further insights into the system, but the methods used here served well to address

the questions designated at the outset of the study.

6.7 Future work

6.7.1 Weathering under natural conditions with constant immigration

This study was a laboratory study using artificial microcosms to study weathering under aerobic and anaerobic conditions. There are several reasons why this was a useful set-up, as it allows the system to be well-controlled in order to answer the questions posed. A discrepancy between this set-up and the natural environment is the lack of immigration into the system, and it may be interesting to set up a weathering study with immigration into the system. To some extent, this was done in the colonisation study (Chapter 3), but without the explicit goal to study weathering in detail, but there is scope for more studies in this direction.

6.7.2 Weathering with smaller artificial communities or isolates

As this was a complex, established microbial community under study in this experiment, it might be interesting to repeat the set-up using a smaller artificial community or individual isolates. These types of studies have been conducted in the past, allowing for some different ways of analysing weathering processes [177], [224]. Here, it is difficult to ascertain exactly which members of the community have an impact on the weathering and vice versa, such that only the broader community effects can be studied. For the intents and purposes of this study, the set-up used was adequate, but there are other insights that could be drawn by using a more tightly controlled community with parameters involved.

6.7.3 Colonisation and weathering under anaerobic conditions

One experiment which would be interesting, albeit difficult, to set up would be to study microbial weathering, colonisation and community assembly under

anaerobic conditions with influx of microorganisms from the atmosphere. This would be an extremely difficult set-up that would require constant flushing of the system with gas. It is however an interesting topic to consider, as it would serve as an early Earth analogue, albeit with a modern pool of organisms, and it would be very intriguing to see what sort of communities would form and how these would increase in complexity over time, and what effect their presence would have on the weathering of the rock substrate. Another way to accomplish this would be to bury or submerge sterile microcosms in an anaerobic environment, where colonisation would be from sediments or water, instead of from aeolian input. This would be a much easier set-up, but perhaps less of an analogy with the early Earth and how the first landmasses were colonised.

6.7.4 Environment-community feedback cycle

One follow-up question from the work conducted here is how the feedback process between microorganisms and the environment affect the emerging complexity. Here, analysis was conducted on the effects of the microbial community on the weathering of the rock substrate, with community composition only analysed at the end of the experiment. It is clear from these results that the microbial community does alter the course of weathering, which would have an effect on the future development of the community, which may have been more obvious in a situation where immigration into the community was constant, rather than a single starting-point inoculation. In principle, it is true in natural communities that there is an effect of microbial weathering on the community itself, and it is possible to identify patterns whereby leaching or influx of different elements can shape the community. In order to understand the long-term effects of microbial weathering, analysis would have to be conducted that would investigate this feedback cycle, where the effect of both the microbial community on weathering, as well as the effect of weathering on the microbial community, would be investigated. The results from this study indicate that these effects are important, and would be useful to investigate.

6.8 Conclusions

In this experiment, the effect of a microbial community on the weathering of a rock substrate under aerobic and anaerobic conditions was studied. This was achieved using laboratory microcosms with a soil microbial inoculum, which were monitored for 7 weeks. Several useful insights into the weathering process and community structure were gained through this study. Firstly, it was found that more elements were readily leached from granite than gabbro, which was surprising as granite in general has a lower abundance of these elements than gabbro, as granite has a higher silica content. Secondly, it was concluded that the main difference between the microbial communities in the various conditions was caused by the presence or absence of oxygen. To some extent, this effect may have been observed because of presumed differences in the starting soil inocula. Distinct differences between the rock types were observed in the aerobic conditions when only the presence or absence of taxa was considered, but this effect was attenuated when the relative abundance of taxa was taken into account, resulting in some overlap between the triplicates in each rock type. The communities were dominated by Proteobacteria, which are good primary colonists of rocky, nutrient-poor environments, with Alpha- and Betaproteobacteria dominating the aerobic communities while Gammaproteobacteria made up the majority of the anaerobic communities. The anaerobic communities are much more dominated by a single taxon than the aerobic communities, which have no single dominant taxa at genus level. It is concluded that the absence of oxygen changes the leaching patterns, with overall less leaching observed in the anaerobic communities, particularly in gabbro, which is consistent with anaerobic metabolisms in general being slower, hence reducing weathering rates. When examining the differences in leaching between weeks 1 and 7, the patterns are similar for the two rock types, but separate when it comes to whether or not the systems are aerobic or anaerobic. It appears that the changes in leaching depend on whether or not it is a system with or without oxygen, and little on the rock substrate. This is assumed to be due to differences in microbial community composition between the aerobic and anaerobic conditions, that have different metabolic functions and hence weathering capabilities. In sum, this study increases our understanding on the topic of microbial weathering by a natural community in the presence and absence of oxygen by addressing a number of different questions, and suggests that microorganisms have an important role to play in the weathering of the Earth's crust.

Chapter 7

Conclusions

Taken together, the four experiments in this work have helped elucidate various aspects of microbial community assembly in rock environments. For the first time, a rigorous time series study has been undertaken to look at how microorganisms colonise a new rock environment over a period of 18 months. This research has implications for how landmasses were first colonised on the early Earth, as well as the colonisation of new habitats that form in the present day, such as fresh lava flows, glacial forefields and areas sterilised or disturbed by wildfire. In addition, the knowledge garnered on community assembly is helpful for understanding how microorganisms colonise new environments in general, which has implications for many areas of life and society as a whole.

Looking at the collective results attained in this project, some overarching points can be made by comparing the results from the various studies. One interesting observation is that some of the effects seen in the priority effects experiment and the weathering experiment are not observed as clearly in the colonisation experiment. Considering the colonisation experiment to the more natural, complex system, the other two experiments are relatively constrained laboratory studies using microcosms, with far fewer variables involved. The lessons from the priority effects chapter are that under certain circumstances, such as those observed here, priority does matter, and the species that arrive into the system first can ultimately grow to larger numbers than those arriving later. In the weathering chapter the results show stark differences in leaching rates between the two rock types, granite and gabbro. Although both these effects can explain some of the results seen in the colonisation experiment, they are not observed as

clearly. The explanation for this may be that the larger, more complex natural system in the colonisation study obscures some of the smaller effects that can be pulled out when studying a smaller system under more constrained conditions.

There are several differences between the set-up in the weathering experiment compared to the colonisation study that would explain differences in leaching, biomass and community composition. The colonisation microcosms had a flow-through system, meaning that organisms or nutrients could disappear from the system, either continuously or at certain events of heavy rain, while the weathering experiment consisted of closed microcosms where nutrients would have to be recycled, and no immigration or escape of organisms from the system could occur. Carbon sources are another factor - in the weathering experiment the main carbon source is supplied as glucose at the onset, whereas in the colonisation system there is no initial carbon source present, but atmospheric carbon has the potential to enter the system at any point, and is over time being supplied by the presence of phototrophs that immigrate into the system. Also, the colonisation set-up experiences fluctuations in temperature, humidity and air pressure, whereas the weathering microcosms are sealed and stored in a static environment, with the only changes occurring within the system. Taken together, these factors can result in vast differences between observations in the two experiments, even though they share some common characteristics.

The fact that the more complex system, the colonisation experiment, seems less sensitive to smaller-scale perturbations or influences has some wider implications. A more complex system typically has more variables that can be altered or fluctuate within the system, while still keeping the overall processes in a steady state. A smaller, less complex system might immediately reveal the effects of a recent perturbation through a cascade effect on observable variables. This means that something like priority effects may still be playing a role in the colonisation experiment, but they may be smoothed out by the myriad of other processes also taking place which simultaneously shape the system. For instance, a complex ecosystem is typically more resistant or resilient to perturbation than a simple system or single species, which can easily be knocked out by adverse conditions or stress.

This observation about complex systems often being more resistant or resilient to change has a general implications for the way experiments are conducted. Many experiments benefit from being conducted on simple systems where variables can be tightly controlled or accounted for, and this is how much modern research

is conducted. Conducting more complex experiments or working with complex natural systems means that it may be much harder to control the system or distinguish signals from environmental noise. The results from this project support a dual approach, where laboratory experiments can inform, increase understanding of and aid interpretation of a natural system, but that ultimately also studying the natural system itself is vital both to understanding the complete picture, and to understanding how the single variables scale up to the larger system. Thus, laboratory experiments are useful for studying specific criteria individually, while field observations give insight into the collective effects. By pursuing both lines of inquiry, results should be both detailed and reliable, ensuring that progress is made.

Some overarching observations are made about the technical aspects of culturing these complex microbial communities on agar plates, that are consistent across the experiments conducted here. Firstly, it is observed that when conducting dilution series, pigmented isolates are only observed when the dilution is high and the agar plates are not overgrown. It is unclear whether it is the same organisms growing at low dilutions as at high dilutions, and the pigments only being expressed at high dilutions, or whether there are different organisms growing, and the pigmented organisms being outcompeted at low dilutions. Either way, it is a consistent finding in these experiments that pigmented colonies are only observed when the dilution is high enough so that each colony is spatially separated on the plate, whereas when the colonies overlap or grow to cover the entire agar surface, all colonies are either white, clear or buff coloured. This phenomenon does not appear to be well studied or described in the existing literature, and could be an interesting topic for further investigation. Secondly, it was observed that dilution series of the communities often does not lead to an accurate proportional drop in CFUs. In other words, a ten-fold dilution is seldom found to lead to a ten-fold decrease in CFU numbers on a plate, meaning that CFU counts are not completely reliable for these complex communities when dilutions have to be performed, and that it is difficult to compare growth when different samples have to be diluted by different amounts. This effect could be due to heterogeneity in the distribution of the various taxa in the liquid, or in the case of the experiment addressing priority effects, that cells were clumping when growing in liquid culture. Heterogeneity in the spatial distribution of certain taxa within a community could mean that dilution series are not reliable, as the proportion of the various species that are transferred in a dilution is not consistent, and that when the dilution series is spread on an agar plate, different species that can grow well on agar plates are

present in different proportions. Thus, this could explain observations that for the community as a whole, dilution series do not lead to proportional decreases in total growth of the community on agar plates. Again, this topic is not well covered in existing literature, and would warrant further investigation. Thus, this body of work points to some avenues for future research with implications for the methodology of common culturing techniques.

In sum, this body of work adds substantially to our knowledge of microbe-mineral systems, specifically related to how microbial communities are assembled and colonise new environments, and how complexity emerges in these systems. The first rigorous time series study of the natural process of colonisation by microorganisms of fresh rock was set up, and the system has been described in detail, over the course of 18 months. This experiment lends support to the idea that neutral processes and species sorting are both important processes in community assembly, and that community structure is influenced by both time and rock substrate, where rock substrate is a strong enough selection factor to create distinction in community composition under otherwise identical environmental conditions. A nascent and pioneering approach is adopted, whereby genetic sequencing data is combined with quantification of the amount of gene copies present in the samples, allowing for the absolute abundances of detected taxa to be determined. The effect of environmental stress in terms of freeze-thaw cycles on a microbial community was investigated in another experiment, with results supporting the idea that communities are better at withstanding environmental stress earlier in the community assembly process, potentially because of the dominance by hardy pioneer species. In a third study, the role of priority in microbial community assembly was investigated, showing that for environmental isolates with similar growth rates, the order of colonisation has a direct effect on the growth and biomass of the species in the system. Lastly, an experiment was set up to study how microbial communities can affect environmental and geochemical change, by analysing microbial rock weathering under aerobic and anaerobic conditions, showing that both rock type and the presence or absence of oxygen create unique leaching patterns. These studies reveal some of the processes by which microbial communities are formed and colonise a new environment. Microbial communities form rapidly when a new pristine environment appears, and experience changes in community composition over time, as pioneer species pave the way for secondary colonists. Microbial communities alter the geochemical environments in which they take up residence, and changes in the environment can also drive corresponding

alterations in the community structure. Environmental stress has an impact on community biomass and structure over short time scales, but over time without major disturbance the community composition changes gradually rather than through sudden major shifts. The order in which species colonise a new environment can affect community composition, and can be partly responsible for the differences observed in community composition among microcosms with identical conditions, where communities are broadly similar but distinct. The type of rock substrate on which communities form helps shape the community and different rocks appear to have distinct communities, but under otherwise identical environmental conditions and immigration processes, microbial communities in different rock types have similar overall structures, especially at higher taxonomic levels. Thus, rock type and geochemical environment shapes the microbial community when a new habitat is colonised. Microbial community assembly is a complex and dynamic process, which under natural conditions is shaped by a myriad of factors. Some of the processes of community assembly can be teased apart under laboratory experiments, while studies of the whole natural system reveal more information on the larger scales, and thus a dual approach helps probe the phenomenon from various angles. Together, these investigations help us better understand how microbial communities are formed, and how they shape and alter the environment around them.

Appendix A

Sample grid layout for colonisation experiment

	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	
	161	183	206	21	207	96	78	4	64	85	134	26	148	30	165	111	80	81	
	136	190	139	153	45	181	66	39	137	60	11	52	83	189	186	144	152	10	
	169	28	82	7	91	193	75	149	76	101	17	95	131	5	173	19	90	117	
	130	158	185	37	174	65	212	125	44	138	42	213	61	116	164	147	142	202	
	215	151	178	109	108	53	198	140	201	22	99	73	184	23	16	35	49	133	
	33	208	51	156	97	25	102	68	57	192	6	86	79	127	180	24	204	195	
	122	163	98	32	187	121	199	1	72	43	120	46	126	9	20	58	123	188	
	100	29	124	167	128	12	14	129	69	77	113	166	3	93	88	27	196	50	
	70	63	205	146	132	13	177	182	170	62	8	150	110	171	106	71	143	191	
	115	197	31	48	34	36	211	47	15	2	176	67	168	114	154	216	40	172	
	18	141	175	92	203	112	145	155	105	94	119	200	194	210	135	54	159	74	
	87	89	38	41	84	59	179	103	56	55	157	107	118	214	162	209	160	104	
	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	
	253	254	255	256	257	258	259	260	261	262	263	264							

1-3-16 Final roof layout

Figure A.1 *Sample grid layout for the colonisation experiment as described in Chapter 3.*

1	Gr	tp1	
2	Gr	tp1	
3	Gr	tp1	
4	Gr	tp1	
5	Gr	tp1	
6	Gr	tp1	
7	Gr	tp1	
8	Gr	tp1	
9	Gr	tp1	
10	Gr	tp2	
11	Gr	tp2	
12	Gr	tp2	
13	Gr	tp2	
14	Gr	tp2	
15	Gr	tp2	
16	Gr	tp2	
17	Gr	tp2	
18	Gr	tp2	
19	Gr	tp3	
20	Gr	tp3	
21	Gr	tp3	
22	Gr	tp3	
23	Gr	tp3	
24	Gr	tp3	
25	Gr	tp3	
26	Gr	tp3	
27	Gr	tp3	
28	Gr	tp4	
29	Gr	tp4	
30	Gr	tp4	
31	Gr	tp4	
32	Gr	tp4	
33	Gr	tp4	
34	Gr	tp4	
35	Gr	tp4	
36	Gr	tp4	
37	Gr	tp5	
38	Gr	tp5	
39	Gr	tp5	
40	Gr	tp5	
41	Gr	tp5	
42	Gr	tp5	
43	Gr	tp5	
44	Gr	tp5	

45	Gr	tp5	
46	Gr	tp6	
47	Gr	tp6	
48	Gr	tp6	
49	Gr	tp6	
50	Gr	tp6	
51	Gr	tp6	
52	Gr	tp6	
53	Gr	tp6	
54	Gr	tp6	
55	Gr	tp7	
56	Gr	tp7	
57	Gr	tp7	
58	Gr	tp7	
59	Gr	tp7	
60	Gr	tp7	
61	Gr	tp7	
62	Gr	tp7	
63	Gr	tp7	
64	Gr	tp8	
65	Gr	tp8	
66	Gr	tp8	
67	Gr	tp8	
68	Gr	tp8	
69	Gr	tp8	
70	Gr	tp8	
71	Gr	tp8	
72	Gr	tp8	
73	Gr	tp9	
74	Gr	tp9	
75	Gr	tp9	
76	Gr	tp9	
77	Gr	tp9	
78	Gr	tp9	
79	Gr	tp9	
80	Gr	tp9	
81	Gr	tp9	
82	Gr	tp10	
83	Gr	tp10	
84	Gr	tp10	
85	Gr	tp10	
86	Gr	tp10	
87	Gr	tp10	
88	Gr	tp10	

89	Gr	tp10	
90	Gr	tp10	
91	Gr	tp11	
92	Gr	tp11	
93	Gr	tp11	
94	Gr	tp11	
95	Gr	tp11	
96	Gr	tp11	
97	Gr	tp11	
98	Gr	tp11	
99	Gr	tp11	
100	Gr	tp12	
101	Gr	tp12	
102	Gr	tp12	
103	Gr	tp12	
104	Gr	tp12	
105	Gr	tp12	
106	Gr	tp12	
107	Gr	tp12	
108	Gr	tp12	
109	Ga	tp1	
110	Ga	tp1	
111	Ga	tp1	
112	Ga	tp1	
113	Ga	tp1	
114	Ga	tp1	
115	Ga	tp1	
116	Ga	tp1	
117	Ga	tp1	
118	Ga	tp2	
119	Ga	tp2	
120	Ga	tp2	
121	Ga	tp2	
122	Ga	tp2	
123	Ga	tp2	
124	Ga	tp2	
125	Ga	tp2	
126	Ga	tp2	
127	Ga	tp3	
128	Ga	tp3	
129	Ga	tp3	
130	Ga	tp3	
131	Ga	tp3	
132	Ga	tp3	

133	Ga	tp3	
134	Ga	tp3	
135	Ga	tp3	
136	Ga	tp4	
137	Ga	tp4	
138	Ga	tp4	
139	Ga	tp4	
140	Ga	tp4	
141	Ga	tp4	
142	Ga	tp4	
143	Ga	tp4	
144	Ga	tp4	
145	Ga	tp5	
146	Ga	tp5	
147	Ga	tp5	
148	Ga	tp5	
149	Ga	tp5	
150	Ga	tp5	
151	Ga	tp5	
152	Ga	tp5	
153	Ga	tp5	
154	Ga	tp6	
155	Ga	tp6	
156	Ga	tp6	
157	Ga	tp6	
158	Ga	tp6	
159	Ga	tp6	
160	Ga	tp6	
161	Ga	tp6	
162	Ga	tp6	
163	Ga	tp7	
164	Ga	tp7	
165	Ga	tp7	
166	Ga	tp7	
167	Ga	tp7	
168	Ga	tp7	
169	Ga	tp7	
170	Ga	tp7	
171	Ga	tp7	
172	Ga	tp8	
173	Ga	tp8	
174	Ga	tp8	
175	Ga	tp8	
176	Ga	tp8	

177	Ga	tp8	
178	Ga	tp8	
179	Ga	tp8	
180	Ga	tp8	
181	Ga	tp9	
182	Ga	tp9	
183	Ga	tp9	
184	Ga	tp9	
185	Ga	tp9	
186	Ga	tp9	
187	Ga	tp9	
188	Ga	tp9	
189	Ga	tp9	
190	Ga	tp10	
191	Ga	tp10	
192	Ga	tp10	
193	Ga	tp10	
194	Ga	tp10	
195	Ga	tp10	
196	Ga	tp10	
197	Ga	tp10	
198	Ga	tp10	
199	Ga	tp11	
200	Ga	tp11	
201	Ga	tp11	
202	Ga	tp11	
203	Ga	tp11	
204	Ga	tp11	
205	Ga	tp11	
206	Ga	tp11	
207	Ga	tp11	
208	Ga	tp12	
209	Ga	tp12	
210	Ga	tp12	
211	Ga	tp12	
212	Ga	tp12	
213	Ga	tp12	
214	Ga	tp12	
215	Ga	tp12	
216	Ga	tp12	
217	Gr	tp1	dc
218	Gr	tp1	wc
219	Gr	tp2	dc
220	Gr	tp2	wc

221	Gr	tp3	dc
222	Gr	tp3	wc
223	Gr	tp4	dc
224	Gr	tp4	wc
225	Gr	tp5	dc
226	Gr	tp5	wc
227	Gr	tp6	dc
228	Gr	tp6	wc
229	Gr	tp7	dc
230	Gr	tp7	wc
231	Gr	tp8	dc
232	Gr	tp8	wc
233	Gr	tp9	dc
234	Gr	tp9	wc
235	Gr	tp10	dc
236	Gr	tp10	wc
237	Gr	tp11	dc
238	Gr	tp11	wc
239	Gr	tp12	dc
240	Gr	tp12	wc
241	Ga	tp1	dc
242	Ga	tp1	wc
243	Ga	tp2	dc
244	Ga	tp2	wc
245	Ga	tp3	dc
246	Ga	tp3	wc
247	Ga	tp4	dc
248	Ga	tp4	wc
249	Ga	tp5	dc
250	Ga	tp5	wc
251	Ga	tp6	dc
252	Ga	tp6	wc
253	Ga	tp7	dc
254	Ga	tp7	wc
255	Ga	tp8	dc
256	Ga	tp8	wc
257	Ga	tp9	dc
258	Ga	tp9	wc
259	Ga	tp10	dc
260	Ga	tp10	wc
261	Ga	tp11	dc
262	Ga	tp11	wc
263	Ga	tp12	dc
264	Ga	tp12	wc

Figure A.2 *Legend for sample grid layout for the colonisation experiment as described in Chapter 3. Columns are from left-right: sample number (as seen in the grid layout), rock type, time point (=tp), and for controls either wc=wet control, dc=dry control.*

Appendix B

Qiime pipeline

A Basic Qiime Workflow

Open Qiime environment

Type `source activate qiime1` into terminal

Validate mapping file

Rename the metadata file from `Cockell_4648B.txt` to `Cockell_4648B_mapping.txt`

```
validate_mapping_file.py -m Cockell_4684BFasta/Cockell_4684B_mapping.txt
-o Cockell_4684BFasta/Cockell_4684B_mapping_corrected
```

Convert fasta file

```
convert_fastaqual_fastq.py -f Cockell_4684BFasta/Cockell_4684B.fna
-q Cockell_4684BFasta/Cockell_4684B.qual
-o Cockell_4684BFasta/Cockell_4684B.fastq
```

Quality control and Qiime preparation

```
extract_barcodes.py
-f Cockell_4684BFasta/Cockell_4684B.fastq/Cockell_4684B.fastq
-l 8 -o Cockell_4684BFasta/Cockell_4684B_barcodes
```

Next step:

```
split_libraries_fastq.py
-m Cockell_4684BFasta/Cockell_4684B_mapping_corrected/
Cockell_4684B_mapping_corrected.txt
-o Cockell_4684BFasta/split_libraries_fastq_Cockell_4684B
-i Cockell_4684BFasta/Cockell_4684B_barcodes/reads.fastq
-b Cockell_4684BFasta/Cockell_4684B_barcodes/barcodes.fastq
-q 19 --barcode_type 8 --phred_offset 33}
```

Combine files from several datasets (all files)

Go into new folder where you move the Fasta folders of the different datasets to.

```
cat combinedtp1-10/combined_seqs.fna
Cockell_5249BFasta/split_libraries_fastq_Cockell_5249B/seqs.fna
> combinedtp1-11/combined_seqs.fna
```

Continue working on the combined file, as follows:

Split file

```
filter_fasta.py
-f combinedtp1-11/combined_seqs.fna
-o combinedtp1-11/filtered_fasta_hanna.fna
--sample_id_fp combinedtp1-11/hanna_seqs_filter_list.txt
```

Chimera removal

```
identify_chimeric_seqs.py
-i combinedtp1-11/filtered_fasta_hanna.fna
-o combinedtp1-11/seqs_checked_chimeras_usearch
-m usearch61 -r Reference_databases/Silva_119_rep_set97.fna
```

```
filter_fasta.py
-f combinedtp1-11/filtered_fasta_hanna.fna
-o combinedtp1-11/filtered_fasta_hanna_chimeras.fna
-s combinedtp1-11/seqs_checked_chimeras_usearch/chimeras.txt -n
```

Pick OTUs

```
pick_open_reference_otus.py
-o combinedtp1-11/Silva119_openref_hanna
-i combinedtp1-11/filtered_fasta_hanna_chimeras.fna
-r Reference_databases/Silva_119_rep_set97.fna

biom summarize-table
-i combinedtp1-11/Silva119_openref_hanna/
otu_table_mc2_w_tax_no_pynast_failures.biom
-o combinedtp1-11/Silva119_openref_hanna/
otu_table_mc2_w_tax_no_pynast_failures_summary.biom
```

Diversity analyses

```
core_diversity_analyses.py
-i combinedtp1-11/Silva119_openref_hanna/
otu_table_mc2_w_tax_no_pynast_failures.biom
-o combinedtp1-11/core_diversity_output_e9400
-m combinedtp1-11/mapping_hanna_corrected.txt
-t Reference_databases/Silva_119_rep_set97_aligned_pfiltered.tre
-e9400 -p combinedtp1-11/alpha_parameters.txt
```

Appendix C

Sample location details, Isle of Skye

Sampling started at the Broadford Gabbro site in the late afternoon of 26 January 2015. This site is a former tree plantation quite heavily covered in vegetation, which is at least in its second phase, with the stumps of mature trees remaining and new shoots planted. Three samples were collected here on the first day, with aerobic samples collected in Whirlpak bags and anaerobic samples collected in anaerobic Duran bottles (250ml), beside a dirt track leading from the Broadford cemetery and up the hill, over a stream and beneath a power line. This track follows an old railroad track with evidence of old sleepers, that ran from the Broadford marble quarry to Broadford pier. The first samples (no.1) were collected by the side of this little track, with samples taken aerobic and anaerobic at site (1) on heather, with quite a lot of vegetation at the surface, and dug to about 20cm down for anaerobic samples. Site (2) was taken aerobically in a small stream using a spade and trowel, further up the hillside. Some rocks were collected between site (1) and (2) and put in sterile bags, but were not used for further analysis. Further up the small track a path leading to a section with almost bare dirt was found, so samples (3) were collected here. For all sites, aerobic and anaerobic samples were taken in same place, by digging at the surface the aerobic samples and about 20cm down for anaerobic.

Granite was sampled the following day, the first stop being by the drive to the Skye Picture House, between Dunan and Ard Dorch. Sample (4, aerobic only) was collected on rock by the Skye Picture House, on the west side of road. Another sample (5, aerobic only) was collected in a stream near the road by Skye Picture House. The next stop was around the tip of the peninsula, between Luib and the

waterfall. Sample (6) was collected on the south side of the road (A87), on a slope before the switchbacks start, after the top of the peninsula, on an exposed erosion side slope with a biofilm-type carpet layer in green covering the soil. Both aerobic and anaerobic samples were collected at (6). There was no vegetation cover at this site. The next stop was further up the A87 by the popular lookout over the Loch Ainort bay, and took samples (7) in the bank next to the waterfall (LHS when facing the falls), backtracking along the road to the waterfall. There was some vegetation cover, and both aerobic and anaerobic samples were taken here. Riverine samples (8) were collected in the stream Allt na Luibe, by doubling back a bit to somewhere between (6) and (7), and collected both aerobically and anaerobically on a part of the riverbed which was at the time not covered in water, but very close to the waters edge. The next set of samples was collected on a small single-car dirt track along the north side of Loch Ainort. Samples (9) were collected aerobically and anaerobically on a slope on the north side of Loch Ainort, on an erosion type cliff side, similarly to sample (6). The soil was bare, with no vegetation cover, but contained some purple-white fungus or leachate. A metre or two higher up on the slope another aerobic sample (9a) was collected.

After the successful granite sampling, some more samples were collected at the southern end of the Broadford gabbro site, by the track over the cattle guard towards the Allt A Choire guesthouse, past the electric substation near Broadford. Sample (10) was collected aerobically here about 100m up the hill by a rocky outcrop, with characteristic banding or veins. Sample (11) was collected at the gate in the middle of the Broadford gabbro region at two different locations. The aerobic one was collected half-way up the hill in a clearcutting, with newly planted spruce trees. The anaerobic samples came from further down the hill in a tussock by some birch trees with vegetation on top, near the road, just on the edge of the plantation.

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